Characterization of salvage pathways for byproducts of S-adenosylmethionine metabolism

Undergraduate Research Thesis

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By

John Wildenthal

The Ohio State University

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Project advisor: F. Robert Tabita, Department of Microbiology

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Preface

This study largely focused on the function of a three-gene operon in the metabolism of byproducts from reactions involving S-adenosylmethionine. This operon, consisting of genes encoding a phosphorylase, isomerase, and aldolase, constitute the first three steps in a novel, anaerobic methionine salvage pathway in the model organism *Rhodospirillum rubrum*. In this pathway, these enzymes are used to convert an inhibitory, sulfur-containing byproduct, 5'-methylthioadenosine, into usable forms of sulfur. Furthermore, these enzymes appear to be involved in the salvage of carbon from another inhibitory byproduct, 5'-deoxyadenosine. These two compounds are both byproducts of S-adenosylmethionine metabolism.

In Chapter I, the first two genes in the operon, the phosphorylase and isomerase, are characterized. Chapter II describes the aldolase, which was found to function in a previously unknown anaerobic methionine salvage pathway. Chapter III further describes the characterization of other homologous aldolases from various bacteria in relation to methionine salvage. Chapter IV seeks to extend the salvage function of the phosphorylase, isomerase, and aldolase to the compound 5'-deoxyadenosine. Chapter V describes the methods used in the previous chapters.

Overall, the dual potential of this operon to be used both in methionine salvage and 5'-deoxyadenosine salvage lead it to be interesting, especially when considering anaerobic metabolism. The methionine salvage pathway involving the aldolase only functions under anaerobic conditions, and 5'-deoxyadenosine is also generated at higher levels under anaerobic conditions. The majority of species that contain this operon are also facultative or obligate anaerobes, further underpinning the potential importance of this operon in anaerobic metabolism.

Chapter I: Examination of the role of MTAP and MTRI in aerobic and anaerobic methionine salvage in *R. rubrum*

Introduction

Sulfur is an essential element that is required for numerous metabolic functions in organisms. Sulfur must be assimilated by cells and maintained in the proper oxidation state for the synthesis of necessary sulfur-containing metabolites. Such metabolites include the amino acids cysteine and methionine, which are further required for the synthesis of proteins, glutathione, Coenzyme A, and S-adenosylmethionine (SAM)[1,18]. When SAM is used in polyamine synthesis for cellular growth, homoserine lactone production for quorum sensing, or ethylene formation in plants for use as a hormone, 5'-methylthioadenosine (MTA) is formed[1]. MTA is a substrate level inhibitor of SAM-utilizing enzymes, and thus, accumulation can inhibit cellular growth[18,19]. MTA also contains sulfur. Given that biologically accessible sulfur can be limiting in certain environments, such as freshwater lakes and rivers[12], it can be beneficial for organisms to recycle the MTA into a non-inhibitory, usable form of sulfur, generally methionine. This process is undertaken by methionine salvage pathways (MSPs) that are present in many types of organisms[1,5,12].

The first methionine salvage pathway that was discovered, the Universal Methionine Salvage Pathway, exists in most eukaryotes and many prokaryotes[1]. This salvage pathway uses a phosphorylase, isomerase, dehydratase, enolase/phosphatase, dioxygenase, and transaminase to sequentially convert MTA back into methionine (Figure. 1, brown pathway)[1]. Notably, the dioxygenase enzyme (MtnD) requires the use of molecular oxygen as a substrate. Due to this

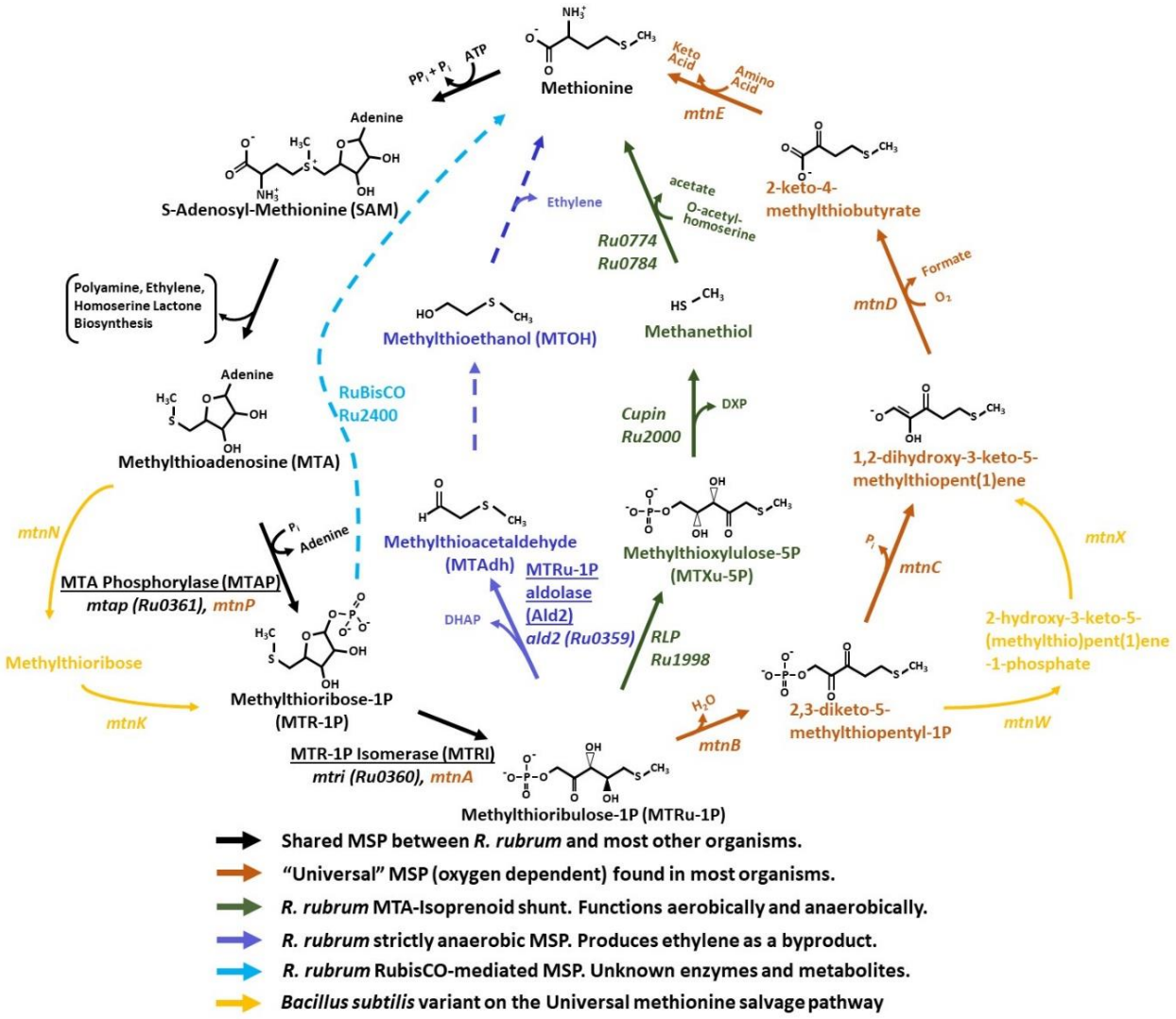

Figure 1: Methionine salvage pathways. In brown, the "Universal" methionine salvage pathway found in most eukaryotes and many prokaryotes. In green, the MTA-isoprenoid shunt of *R. rubrum*. In dark blue, the *R. rubrum* strictly anaerobic methionine salvage pathway that creates ethylene as a byproduct. In light blue, the *R. rubrum* RuBisCO-mediated methionine salvage pathway. In yellow, the *Bacillus subtilis* variant of the Universal methionine salvage pathway

step, the Universal MSP can only function under aerobic conditions. However, the existence of anaerobic functioning MSPs have been long postulated[1].

Recently, the first oxygen-independent MSP in *Rhodospirillum rubrum*, a metabolically-diverse, photoautotrophic alphaproteobacterium from the purple non-sulfur family. This pathway, the "MTA-isoprenoid shunt," functions under both aerobic and anaerobic conditions (Figure 1, green pathway)[2-5]. This pathway shares two enzymatic steps with the Universal MSP. Similar to the Universal MSP, a phosphorylase (MTAP) cleaves the adenine group from MTA and replaces it with a phosphate, generating 5-methylthioribose-1-phosphate (MTR-1P)[5]. Then, an isomerase enzyme (MTRI) isomerizes MTR-1P into 5-methylthioribulose-1-phosphate (MTRu-1P)[5]. Diverging from the Universal MSP, the MTA-isoprenoid shunt then uses a RuBisCO like protein (RLP) from the Deep-YkrW clade to isomerize MTRu-1P into a 3:1 mixture of 1-methylthioxylulose-5-phosphate (MTXu-5P) and 1-methylthioribulose-5-phosphate (MTRu-5P)[3]. The MTXu-5P is then acted upon by a cupin-superfamily methylsulfurylase (Cupin, Rru_A2000) which cleaves MTXu-5P into methanethiol and 1-deoxyxylulose-5-phosphate (DXP)[5]. The methanethiol is then incorporated into methionine via an O-acetyl-homoserine sulfhydrolase enzyme (Rru_0774, Rru_A0784)[5].

The use of RuBisCO-like-proteins in sulfur salvage has long been interesting. Phylogenetic analysis of RuBisCO proteins has determined four main classes, labelled form I, II, III, and IV[3]. Forms I, II, and III all are used to catalyze the carboxylation or oxygenation of ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate (3PGA), or one molecule of phosphoglycolate and one molecule of 3PGA, respectively[3]. However, form IV RuBisCOs, also known as RuBisCO-like-proteins (RLPs), cannot catalyze this canonical reaction due to one or more essential amino acid residue changes within the RubisCO active site

domain[3,26]. The function of RLPs is largely unknown, but two RLPs have been discovered to function in methionine salvage[3,26]. The first, MtnW from *Bacillus subtillis*, is an RLP from the YkrW clade. This RLP is used in a variant of the Universal Methionine Salvage Pathway to tautomerize 2,3-diketo-5-methylthiopentyl-1P (Figure 1, yellow pathway)[3,26]. The second, *R. rubrum*'s RLP from the Deep-YkrW clade, has been discovered to isomerize MTRu-1P to MTXu-5P and MTRu-5P as part of the MTA-isoprenoid shunt described above (Figure 1, green pathway). Conversely, bona-fide form I, II, and III RuBisCOs are incapable of catalyzing these reactions with the respective substrates[35].

Remarkably, it was discovered that the MTA-isoprenoid shunt, wherein the Deep-YkrW RLP participates, was not the only methionine salvage pathway present in *R. rubrum*. When the *R. rubrum* RLP enzyme was inactivated, the organism could not utilize MTA as the sole sulfur source under aerobic conditions[3]. However, under anaerobic conditions, the organism could grow, suggesting the presence of one or more requisite-anaerobic MSPs in *R. rubrum*[3].

Interestingly, when the bona-fide form II RuBisCO of *R. rubrum* (Rru_A2400) was knocked-out, poor to no growth on MTA as the sole sulfur source was observed anaerobically[3]. This implied some essential function of RuBisCO in anaerobic methionine salvage. RuBisCO's potential importance is underlined by the fact that it is upregulated 10-fold at the transcript and protein level when grown on MTA as compared to ammonium sulfate[5]. As the MTA-isoprenoid shunt still appears to be active in the RuBisCO deletion strain (J.A.N. unpublished observations), it has been hypothesized that the RuBisCO deletion may be inhibiting growth either directly by leading to the buildup of an inhibitory metabolite related to methionine salvage, or indirectly by causing a detrimental redox and/or ribulose-1,5-bisphosphate imbalance when cells are grown on MTA versus ammonium sulfate.

This study utilized gene knockouts of the *mtap* and *mtri* genes in order to determine what reaction(s), if any, they catalyzed in the unknown anaerobic MSP(s). Determination of the MTAP and MTRI requirement for anaerobic MTA metabolism would indicate at which step(s) the unknown methionine salvage pathway(s) diverged from the known MTA-isoprenoid shunt. Based on the knockout studies, it was determined that MTAP is required for all methionine salvage in *R. rubrum*, and MTRI is required for aerobic methionine salvage, but not anaerobic methionine salvage. This suggests that at least one anaerobic methionine salvage pathway can act upon MTR-1P independently of the MTA-isoprenoid shunt.

Furthermore, *mtri* was deleted in the ΔRuBisCO/NifA(M173V) background, strain I19*. As discussed earlier, one possible model was that the RuBisCO deletion was leading to the buildup of some unknown, inhibitory methionine-salvage related metabolite(s) originating from MTRu-1P. Elimination of MTRu-1P via elimination of MTRI would determine whether the formation of MTRu-1P was necessary for the production of such a compound. Since MTRI is not required for at least one of the unknown anaerobic pathways, elimination of the *mtri* gene would theoretically restore growth in the RuBisCO knockout strain if MTRu-1P synthesis was necessary for synthesis of the proposed inhibitory metabolite(s) upon which RuBisCO acts. The deletion of *mtri* in the ΔRuBisCO/NifA(M173V) background did not restore growth on MTA as the sole sulfur source, suggesting that if RuBisCO directly functions in methionine salvage, it diverges from the MTA-isoprenoid shunt at the level of MTR-1P.

Detailed materials and methods for Chapter I are given in Chapter V, Section I.

Results

To determine if either MTAP or MTRI were required for methionine salvage in *R. rubrum*, the respective knockout strains, Δ*mtap* and Δ*mtri*, were grown with either 1 mM ammonium sulfate or 0.25 mM MTA added as the sole sulfur source. Growth on MTA would thus suggest the presence of a functional MSP under the given conditions. In order to test the model in which MTRI is involved in the production of inhibitory compounds requiring RuBisCO for further metabolism, the I19* and I19*/Δ*mtri* strains were grown under similar conditions. The results of the growth experiments are shown in Figure 2.

The Δ*mtap* strain was unable to utilize MTA as the sole sulfur source under aerobic or anaerobic conditions, suggesting that conversion of MTA to MTR-1P by MTAP is a required step in all methionine salvage pathways in *R. rubrum*. The Δ*mtri* strain was unable to utilize MTA as the sole sulfur source under aerobic conditions, but could support growth under anaerobic conditions. This suggests that at least one anaerobic MSP can utilize MTR-1P in some unknown capacity to salvage sulfur without using the MTR-1P isomerase gene. Complementation of the Δ*mtap* strain with *mtap* led to restored anaerobic growth on MTA as the sole sulfur source[12]. Complementation of *mtap* and *mtri* genes under aerobic conditions was not performed as previous studies had characterized their function via knockout metabolomics[4].

The I19*Δ*mtri* strain did not restore full growth on MTA, suggesting that MTRu-1P is not required for the hypothesized RuBisCO-linked inhibition of growth. Rather, if RubisCO is required to further process an inhibitory compound(s) resulting from MTA metabolism, such a compound(s) would have to arise from the proposed pathway that diverges from the MTA-isoprenoid shunt after the formation of MTR-1P.

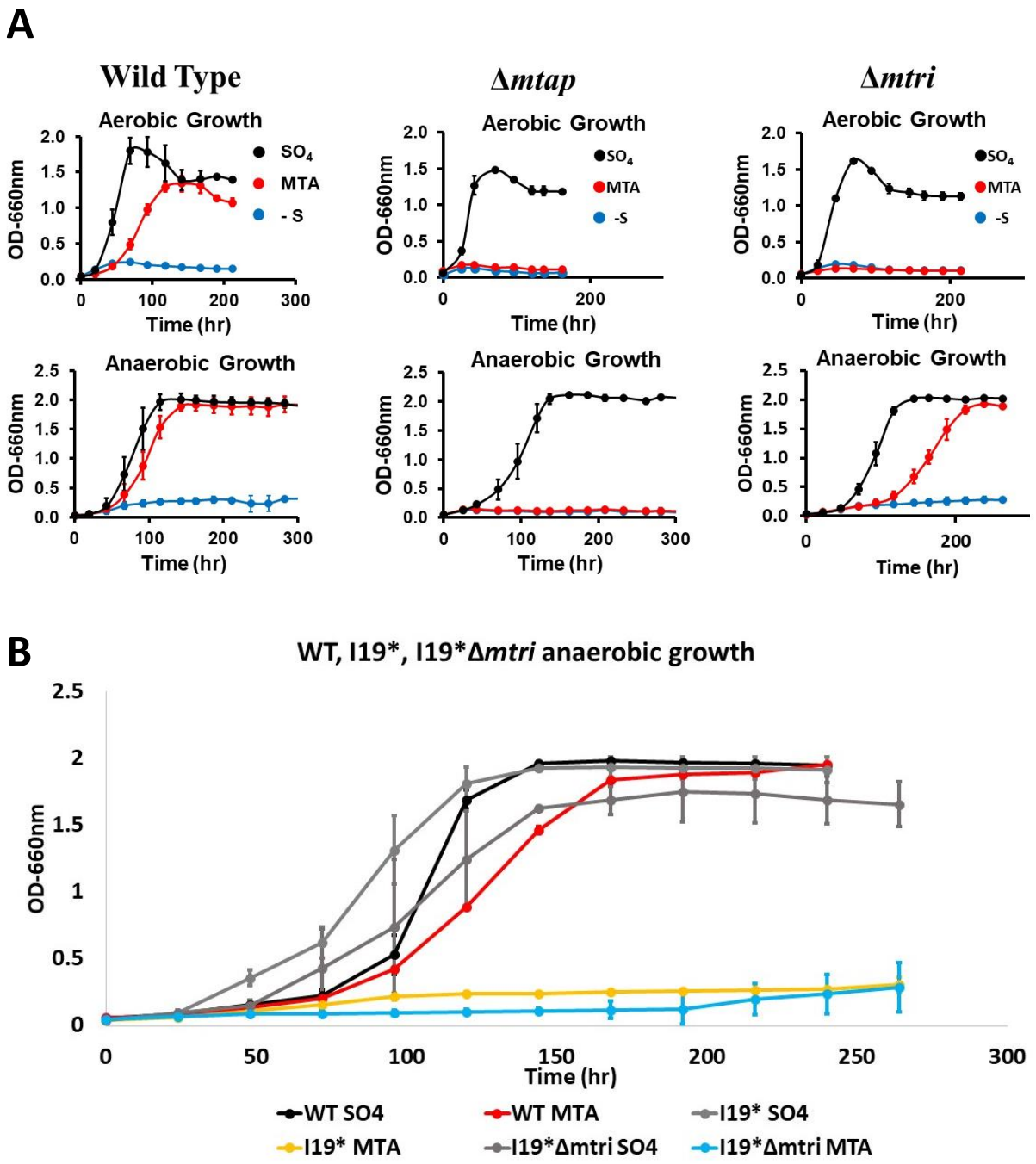

Figure 2: Growth of *R. rubrum* strains. (A) growth of *R. rubrum* Wild Type (WT), Δ*mtap*, and Δ*mtri* strains on MMM with 1 mM ammonium sulfate, 0.25 mM MTA, or no sulfur under aerobic and anaerobic conditions. (B) growth of *R. rubrum* Wild Type, I19* (ΔRuBisCO/NifA(M173V)), and I19*Δ*mtri* strains upon 1mM ammonium sulfate or 0.25 mM MTA under anaerobic conditions.

Discussion

The growth studies done in this experiment give a deeper insight into the diversity of methionine salvage in *R. rubrum*. While formation of MTR-1P by the MTAP enzyme is required for all methionine salvage pathways in *R. rubrum*, the formation of MTRu-1P catalyzed by MTRI does not appear to be required under anaerobic conditions. Under aerobic conditions, both MTAP and MTRI were required for growth. These results, along with previous knockout studies[3,5] suggest that *R. rubrum* contains a single aerobic MSP, and multiple anaerobic MSPs with at least two distinct pathways that can utilize MTR-1P.

Furthermore, deletion of MTRI in the I19* background failed to restore growth. Under the assumption that RuBisCO directly acts upon an inhibitory metabolite associated with growth on MTA, this would mean such a metabolite is not produced via activity of MTRI. Rather, if it were the buildup of an inhibitory metabolite for which RuBisCO is required to further metabolize, it must then arise in the unknown pathway(s) diverging from the MTA-isoprenoid shunt at the level of MTR-1P (Figure. 1, light blue pathway). Previous metabolic studies have determined that this unknown pathway may lead to the formation of S-methyl-cysteine and methylthiolactate[5], but further studies have not been able to directly confirm this (J.A.N. unpublished observations).

While this study indicates that there is likely a second MSP that diverges from the MTA-isoprenoid shunt after the formation of MTR-1P by MTAP, it was hypothesized that other anaerobic MSPs may also exist in *R. rubrum*. Indeed, another such anaerobic MSP is presented in Chapter II.

Chapter II: Characterization of a novel MTRu-1P aldolase from *R. rubrum*

Introduction

It was noted that there was a gene (*ald2*, encoding Ald2) in the same gene cluster as *mtap* and *mtri* genes, which was originally annotated as *mtnB*[25]. The *mtnB* gene product, an MTRu-1P dehydratase, is typically found in organisms containing the Universal MSP[1]. Upon further sequence homology analysis, this gene was re-annotated by the National Center for Biotechnology Information (NCBI) as a class-II aldolase[28], and it shows significant similarity to fuculose-phosphate aldolase gene from *E. coli* (*fucA*, 52% identity; E-value = 2e-65)[27]. Due to its proximity with other known methionine salvage genes in *R. rubrum* and its annotation as a methionine salvage enzyme, it was hypothesized that this gene might participate in one of the unknown methionine salvage pathways in *R. rubrum*. As *R. rubrum* does not contain any genes homologous to Universal MSP genes after *mtnB*, it was postulated that the function of this gene product would be part of a novel methionine salvage pathway. In order to test this, a knockout of the *ald2* gene was constructed in the Wild Type (WT) and Δ*rlp* backgrounds to construct the Δ*ald2* and Δ*rlp*Δ*ald2* strains.

It was hypothesized that, due to gene context and putative function as an aldolase, Ald2 might potentially act upon MTRu-1P as part of an anaerobic methionine salvage pathway in *R. rubrum*. However, because MTRI is not required for anaerobic methionine salvage, presumably any downstream gene products would also not be absolutely required for methionine salvage. Thus, growth phenotypes could not be utilized to determine whether the *ald2* gene was involved in methionine salvage.

Instead, metabolite analysis using [^{14}C] labeled MTA was conducted to determine the function of Ald2 in methionine salvage. While I could contribute to the maintenance of strains, preparation of enzymatic systems, and data analysis, all handling of radioactive materials was done by a postdoctoral fellow, Justin North, who was certified for radioactive work.

In order to detect potential metabolites formed by the *ald2* gene product, the metabolic products of the Δ*rlp* and Δ*rlp*Δ*ald2* strains were compared. The Δ*rlp* background was chosen so that the MTA-isoprenoid shunt beyond the formation of MTRu-1P was inactivated, potentially increasing the metabolic flux through the proposed MSP in which the *ald2* gene product participates. Observation of radiolabeled metabolites present in the Δ*rlp* strain and absent in the Δ*rlp*Δ*ald2* strain would suggest Ald2 function in methionine salvage.

Using this method, a metabolite consistent with 2-methylthioethanol (MTOH) was found to be present in the Δ*rlp* strain and absent in the Δ*rlp*Δ*ald2* strain, suggesting that deletion of *ald2* halted natural cellular production of MTOH. Due to the sequence homology shared between the *R. rubrum ald2* and *E. coli fucA*, a class-II aldolase, the Ald2 enzyme would likely catalyze cleavage of MTRu-1P between the α and β carbons relative to the carbonyl group[29]. The resulting products would be methylthioacetaldehyde (MTAdh) and dihydroxyacetone phosphate (DHAP). The observed MTOH *in vivo* was likely formed via reduction by an unknown alcohol dehydrogenase of MTAdh.

To verify this proposed reaction catalyzed by Ald2, an *in vitro* reconstitution of the proposed metabolic pathway was utilized. Purified *ald2* gene product was supplied with MTRu-1P, and MTAdh was detected via gas chromatography. Further reaction of the MTAdh with commercial yeast alcohol dehydrogenase reduced MTAdh to MTOH, suggesting endogenous *R. rubrum* alcohol dehydrogenases may be able to act upon MTAdh *in vivo*.

Furthermore, growth experiments using the WT and Δ*ald2* strain suggested that *R. rubrum* could use MTOH as a sole sulfur source, further suggesting that MTOH was an intermediate metabolite in a functional anaerobic MSP to recycle MTA.

Detailed materials and methods for Chapter II are given in Chapter V, Section II.

Results

The results of the cellular feedings are shown in Figures 3C and 3D. While most metabolites were conserved between the Δ*rlp* and Δ*rlp*Δ*ald2* strains, deletion of *ald2* resulted in the loss of a peak with a retention time of approximately 20.7 minutes (Figures 3C and 3D, compound 4). Based on the known mechanisms of class-II aldolase proteins[29], it was hypothesized that the Ald2 protein may be catalyzing cleavage of MTRu-1P between the α and β carbons, which would result in the formation of methylthioacetaldehyde (MTAdh), which could potentially be reduced to methylthioethanol (MTOH).

A standard of MTOH was resolved by HPLC, and was discovered to have a similar retention time as the unknown peak (shown in Figure 3E), suggesting the unknown was MTOH. Δ*mtap* and Δ*mtri* strains were fed with [^{14}C]MTA as well, neither of which produced a peak consistent with MTOH. This suggests that MTAP and MTRI, which together produce MTRu-1P from MTA, are also required for the function of the MSP in which Ald2 participates to form MTOH. Altogether, this is consistent with conclusion that Ald2 utilizes MTRu-1P as a substrate for produce MTAdh and DHAP.

When recombinantly expressed and purified Ald2 was added to MTRu-1P, a compound consistent with MTAdh was detected by gas chromatography (Figure 3A), supporting the hypothesized function of Ald2. Furthermore, it was shown that commercial alcohol

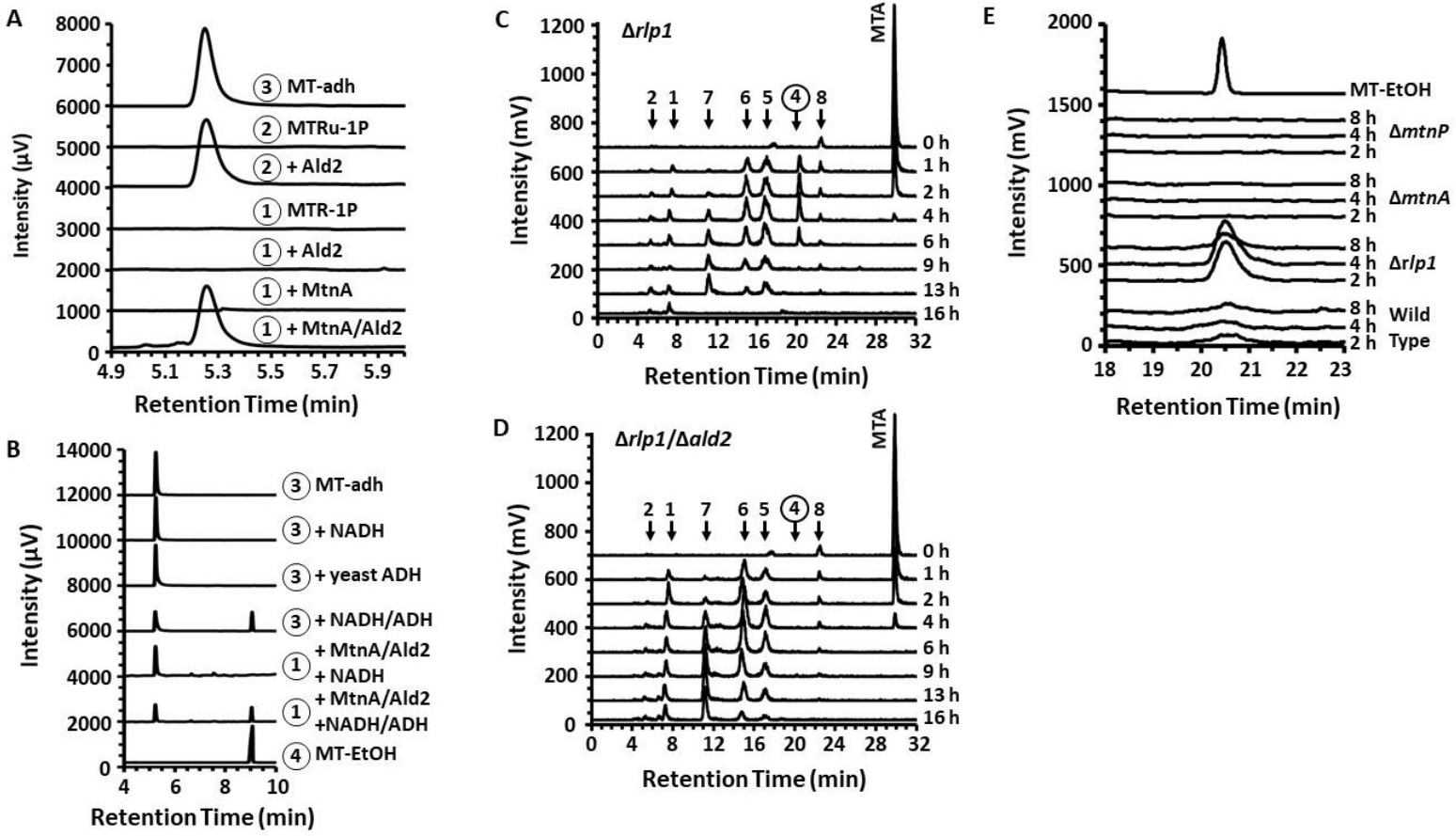

Figure 3: Identification of reaction catalyzed by Ald2. (*A*) GC traces identifying specific conversion of MTRu-1P to MTAdh catalyzed by purified *R. rubrum* Ald2 protein. Conversion of MTR-1P to MTRu-1P is catalyzed by purified *R. rubrum* MTRI. (*B*) GC traces identifying specific conversion of MTAdh to MTOH by yeast ADH. (*C* and *D*) [^{14}C]-Metabolites produced by *R. rubrum* strains Δ*rlp* and Δ*rlp*/Δ*ald2*, respectively, at the indicated time (h) after feeding [methyl-^{14}C]MTA. (*E*) [^{14}C]MTOH observed in the indicated *R. rubrum* strain at the indicated time after feeding [^{14}C]MTA. **Compound list for panels A-D:** (1) 5-methylthioribose-1-P (MTR-1P), (2) 5-methylthioribulose-1-P (MTRu-1P), (3) 2-methylthioacetaldehyde (MT-adh), (4) 2-methylthioethanol (MTOH), (5) 5-methylthioribose (MTR), (6) 5-methylthioribulose (MTRu), (7) unknown not involved in anaerobic ethylene-forming MSP, and (8) unknown contaminant present in commercial [^{14}C]SAM. Figure adapted from North et al 2016.[12]

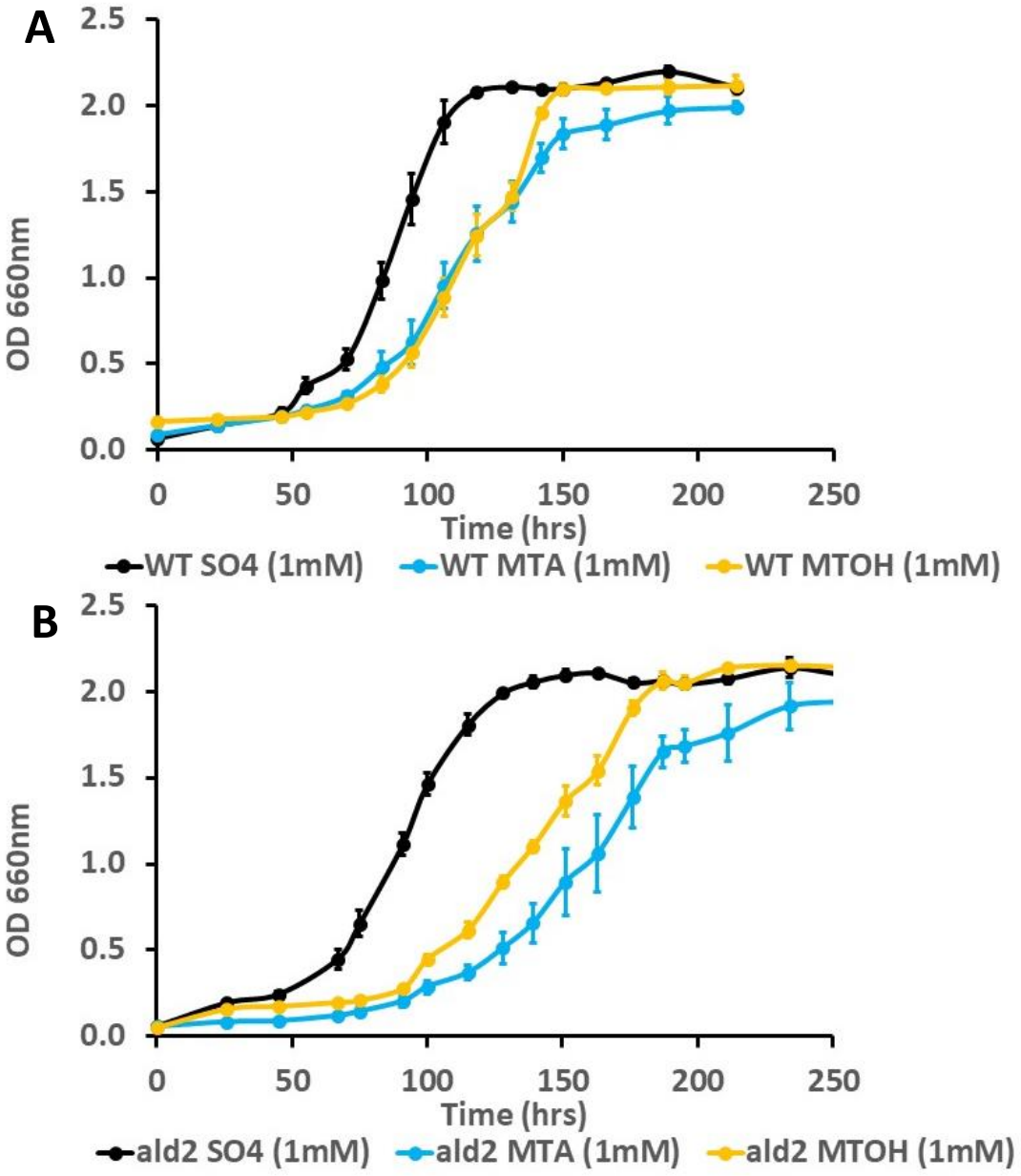

Figure 4: Growth of Wild Type Δ*ald2* strains (A) growth of *R. rubrum* Wild Type on 1 mM ammonium sulfate, 1mM MTA, or 1 mM MTOH as the sole sulfur source under anaerobic conditions. (B) growth of *R. rubrum Δald2* on MMM with 1 mM ammonium sulfate, 1 mM MTA, or 1 mM MTOH as the sole sulfur source under anaerobic conditions.

dehydrogenase from yeast (Sigma) was able to then catalyze reduction of MTAdh into MTOH (Figure 3B), further supporting the idea that an alcohol dehydrogenase results in the *in vivo* formation of MTOH from MTAdh in *R. rubrum*.

To confirm that MTOH was part of a functional MSP, *R. rubrum* Wild Type (WT) and *Δald2* strains were grown using MTOH as the sole sulfur source under aerobic (not shown) and anaerobic conditions (Figure 4). The use of the *Δald2* strain was to prevent any spurious growth phenotypes given by potential reversibility by the Ald2 enzyme (i.e. synthesis of MTRu-1P using MTOH and DHAP followed by metabolism by another existing MSP). Both strains could utilize MTOH as a sole sulfur source anaerobically, and neither could utilize MTOH as a sole sulfur source aerobically. This result, along with the *in vivo* data mentioned above, confirm the role of Ald2 in a novel MSP in *R. rubrum* that functions only under anaerobic conditions.

Discussion

The experiments here demonstrate the capacity of the *R. rubrum* Ald2 to function in a novel, anaerobic methionine salvage pathway (Figure. 1, dark blue pathway). In this pathway, MTRu-1P is cleaved to form DHAP and MTAdh. MTAdh is further converted *in vivo* to MTOH, most likely via the action of an alcohol dehydrogenase. *R. rubrum* has ten annotated alcohol dehydrogenases[25,27,28], and presumably at least one of these has some activity to convert MTAdh to MTOH, given that MTOH is observed *in vivo* and commercial ADH from yeast is able to catalyze the reduction of MTAdh.

Further growth studies confirm that MTOH can be utilized as part of a viable MSP. MTOH can be used as the sole sulfur source in the *ald2* deletion strain, suggesting that there is a further pathway that can metabolize MTOH to usable sulfur such as methionine. This, along with

the enzymatic system showing that MTA can be converted to MTOH, give support to this pathway's function as an anaerobic MSP.

Another interesting facet of this pathway discovered in the Tabita lab concurrently with these experiments was this pathway's link to ethylene biosynthesis. Anaerobic growth on MTA led to a 75-fold increase in natural ethylene excretion from *R. rubrum* WT strain when compared to sulfate, linking methionine salvage to ethylene biosynthesis[12]. Deletion of the *ald2* gene led to a 40-fold reduction in ethylene in cells grown on MTA compared to WT strain under the same conditions, linking ethylene to the specific anaerobic MSP involving *ald2*[12]. Furthermore, when *R. rubrum* strains were fed with small quantities of MTOH, they produced stoichiometric amounts of ethylene gas in a 1:1 ratio[12]. These results suggested that in the process of metabolizing MTOH, ethylene gas is formed as a byproduct. Based on this, the novel anaerobic MSP involving Ald2 was christened the DHAP-ethylene shunt.

Ethylene formation by this MSP has implications in fields such as agriculture and industry. Ethylene gas is used in the manufacturing of plastics such as polyethylene and polyvinyl chloride, and over 158 tons are used in these processes annually[6]. As the vast majority of commercial ethylene production today comes from fossil fuels[6], research into alternative, biological strategies of ethylene production could address concerns of sustainability in these areas.

Ethylene production by soil microbes also has implications in agriculture. Previous studies have detailed anaerobic ethylene production in waterlogged soil[7-9,11]. Ethylene in the soil can inhibit root formation, nodulation, and nitrogen fixation[10,11], and thus can significantly affect crop yields, even at concentrations as low as 1-10 parts per million[10]. While there has been much speculation on the source of anaerobic ethylene production[7-11], the biological sources and

biochemical pathways are still widely unknown. If this strictly anaerobic MSP from *R. rubrum* is prevalent in anoxic soil bacteria, it could potentially explain the anaerobic ethylene production that occurs in waterlogged soils. This would have implications for agriculture, as well as environmental studies in general. Thus, determining potential candidates for a similar strictly anaerobic MSP to the one found in *R. rubrum* could have significant environmental implications.

These applications are further underscored by the potential diversity of this MSP. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG), it was determined that at least 256 organisms had a homologous MTAP, MTRI, and Ald2 to the ones found in *R. rubrum*[12]. Furthermore, 131 of these species had all three genes residing in the same operon, similar to the genomic architecture found in *R. rubrum*[12]. Notably, 83% of the organisms containing all three genes were known facultative or obligate anaerobes, which is in line with the anaerobic nature of this salvage pathway[12]. Chapter III focuses on the characterization of putative Ald2 proteins from some of these organisms in order to determine how widespread this pathway might be.

Chapter III: Construction of an *ald2* complementation system

Introduction

Based on the discovery of a novel ethylene-forming anaerobic MSP in *R. rubrum* (figure. 1, dark blue pathway) and its environmental implications as discussed in Chapter II, we sought to determine if this pathway was functional in other organisms. As the first two enzymes in the novel anaerobic MSP, MTAP and MTRI, are shared with both the Universal MSP and MTA-isoprenoid shunt (Figure. 1, brown and green pathways, repsectivel), they have been well-characterized in many previous studies[1-5,12]. The focus of this study was to characterize putative Ald2 proteins from various bacterial species using a genetic complementation system of transgenic putative *ald2* genes in *R. rubrum*.

The selected strain for this system was the *ΔrlpΔald2* strain. Previous work had discovered that deletion of *rlp* enhanced natural ethylene production when *R. rubrum* was grown on MTA as the sole sulfur source, as this led to more sulfur flux being forced through the DHAP-ethylene shunt[12]. Then, subsequent deletion of the MTR-1P aldolase resulted in near elimination of ethylene production[12]. Thus, the use of this strain would allow for facile detection of ethylene when a functional MTRu-1P aldolase was complemented into the strain.

The base complementation vector used for this experiment was pMTAP, whose construction is detailed in Chapter V, Section I. This vector contains the native *mtap* gene putative promoter element, the natural promoter for the putative operon also containing *R. rubrum*'s *ald2* gene. The start codon of a gene of interest can be cloned into the native transcription start site of *mtap* gene promoter element using an engineered NdeI restriction site.

The putative *ald2* genes chosen for this experiment were from *Rhodopseudomonas palustris, Morganella morganii, Eubacterium limosum,* and *Escherichia coli* ATCC 25922.

R. palustris, similar to *R. rubrum*, is a metabolically diverse alphaproteobacterium and a facultative anaerobe[13]. Furthermore, it has been shown to possess both a functional MTA-isoprenoid shunt and anaerobic ethylene-forming MSP[12]. Its Ald2 is homologous to the one found in *R. rubrum* (Strain CGA010, NCBI locus tag RPA4155; 51% Identity; E: 1e-75 based on NCBI pBLAST)[27], but unlike *R. rubrum*, it is not contained in the same genomic location as *mtap* and *mtri*[28].

M. morganii is a facultative anaerobe from the family *Enterobacteriaceae*[14]. It is an opportunistic pathogen primarily associated with nosocomial infections of the urinary tract[14]. It contains a putative Ald2 homologous to *R. rubrum'*s *ald2* gene product (Strain KT, NCBI locus tag MU9_RS15895; 44% Identity; E: 2e-54 based on NCBI pBLAST)[27]. This aldolase is in the same gene cluster as *mtnK* (encoding MTR kinase, see Figure 1) and *mtri*[28].

E. limosum is an obligate anaerobe from the clostridiacea family[30]. It can utilize CO as the sole carbon/energy source, and thus has potential uses in industry to convert syngas into biofuels[30]. It contains a putative Ald2 homologous to *R. rubrum'*s *ald2* gene product (Strain ATCC 8486, NCBI locus tag RP21_RS02180; 40% Identity; E: 3e-45 based on NCBI pBLAST)[27]. Unlike *R. rubrum*, is not contained in the same gene cluster as *mtap* and *mtri*[28].

E. coli is a facultatively anaerobic gammaproteobacteria that has been widely adapted for industrial uses in gene technology[15]. The ATCC 25922 strain specifically is a clinical isolate that contains two aldolases homologous to the *ald2* gene product[16,28]. The first of these, annotated as a fuculose-phosphate aldolase has 52% Identity (NCBI locus tag DR76_1831, E: 9e-68)[27] with

R. rubrum ald2 based on NCBI pBLAST, and is not contained in the same genomic location as *mtap* and *mtri*[28]. Rather, this aldolase is within the *fuc* operon and is likely the *bona fide* gene *fucA*. The corresponding aldolase enzyme has been labelled *E. coli* FucA for this experiment. The second aldolase, also annotated as a fuculose-phosphate-aldolase, has 44% Identity (NCBI locus tag DR76_2396, E: 1e-52)[27] with *R. rubrum ald2* and is contained in the same genomic location as *mtnK* (MTR kinase) and *mtri*[28]. The corresponding aldolase enzyme has been labelled *E. coli* Ald2 for this experiment.

A schematic showing the genomic context for the aldolase genes from these organisms are presented in Figure 5. Selected *ald2* genes from these organisms were cloned into the pMTAP complementation vector and mated into *R. rubrum* Δ*rlp*Δ*ald2*. Restored ethylene production implied the presence of a functional Ald2 catalyzing the conversion of MTRu-1P to MTAdh. Transgenic aldolases from *R. palustris, M. morganii,* and *E. coli* ATCC 25922 FucA and *E. coli* ATCC 25922 Ald2 all restored ethylene production by the Δ*rlp*Δ*ald2* strain to levels at or above the ethylene production of the *R. rubrum* Δ*rlp* strain in which the native *ald2* gene is still intact.

To determine whether any of these organisms other than *R. rubrum* and *R. palustris* possessed functional MSPs, the type strains were obtained and cultured on defined media with various sulfur sources. *M. morganii* INSali207, *E. limosum* ATCC 8486, and *E. coli* ATCC 25922 were unable to utilize MTA or MTOH as the sole sulfur source, but could grow on either ammonium sulfate, cysteine, and/or methionine. This suggested that either these organisms are incapable of transporting MTA or MTOH inside the cell, or that the putative *mtap*, *mtri*, and *ald2* operon may have a differing function in those organisms unrelated to methionine salvage.

Detailed materials and methods for Chapter III are given in Chapter V, Section III.

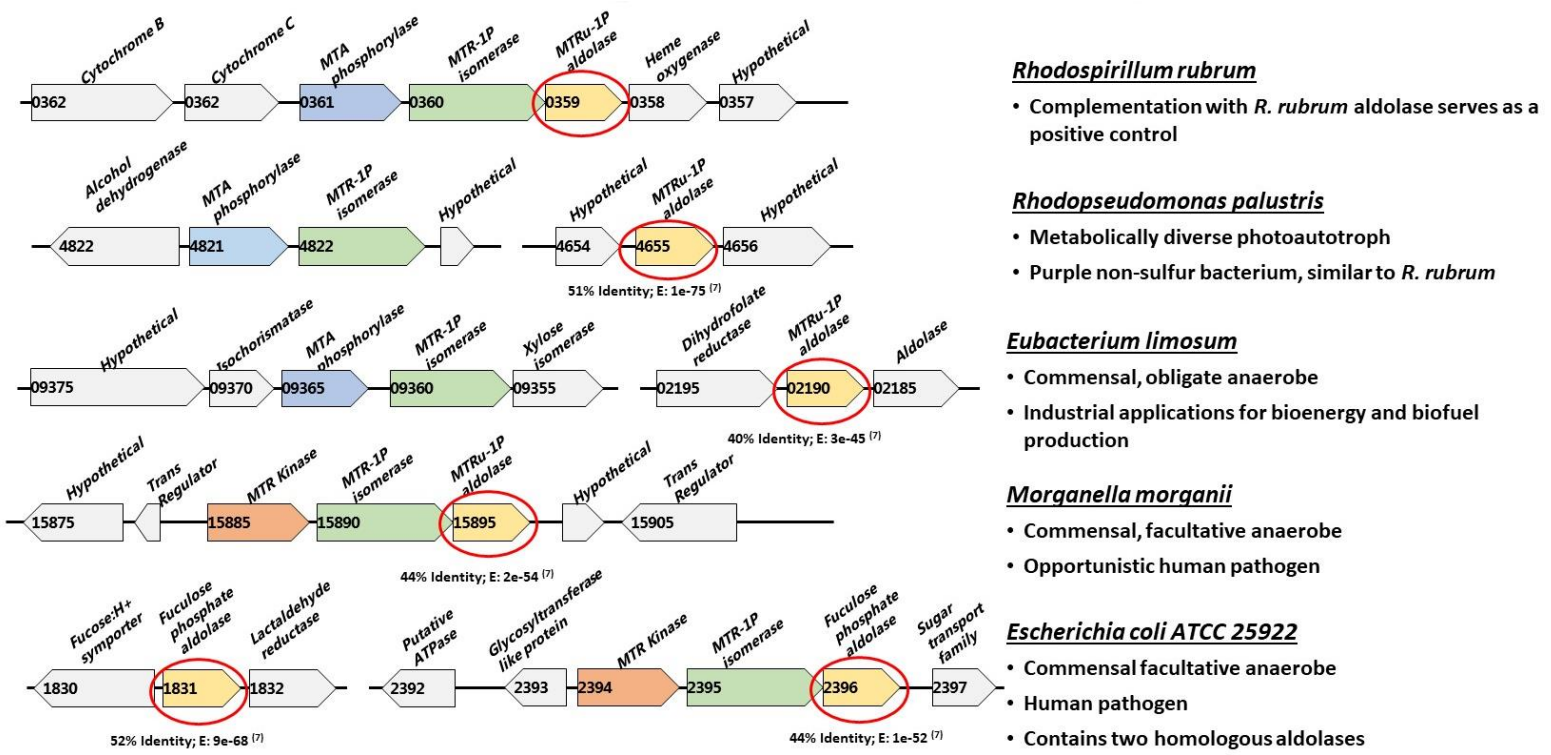

Figure 5: Genomic context of putative *ald2* genes. These genes are from organisms selected for the *ald2* complementation system in *R. rubrum.* Genes are colored as follows: *mtap* in blue, *mtri* in green, *mtnK* in orange, putative *ald2* in yellow and circled red; all other genes are grey. Gene names are based on annotations done by the National Center for Biotechnology Information (NCBI)[28].

Results

Complementation of *R. rubrum* Δ*rlp*Δ*ald2* with the *ald2* genes from *R. rubrum, R. palustris, M. morganii,* and *E. coli* ATCC 25922 all restored ethylene production to levels above or similar to the native ethylene production of *R. rubrum* Δ*rlp* (Figure 5). Notably, the strain with the complemented *ald2* gene from *E. coli* ATCC 25922 produced ethylene at nearly five-fold higher levels than *R. rubrum* complemented with its own *ald2* gene, whereas complementation with the *E. coli fucA* gene only produced roughly the same amount of ethylene as the gene product from *R. rubrum ald2*.

The strains with complemented aldolases from *M. morganii* and *R. palustris* produced ethylene at approximately double the level of the native *ald2* gene product from *R. rubrum*. Complementation with the aldolase from *E. limosum* resulted in partially restored ethylene production, but at five-fold lower levels than that of *R. rubrum* Δ*rlp* or *R. rubrum* Δ*rlp*Δ*ald2* complemented with its own *ald2* gene. These results are detailed in Figure 6.

Growth of organisms on various sulfur sources is shown in Table 1. While *R. rubrum* and *R. palustris* were able to utilize MTA and MTOH as the sole sulfur source, *M. morganii*, *E. limosum,* and *E. coli* ATCC 25922 were unable to utilize these compounds as the sole sulfur source. This suggests that these three organisms may not contain a functional anaerobic MSP similar to the one found in *R. rubrum*. Other potential mechanisms to explain this observed phenotype include a lack of membrane transporters to uptake MTA, requirement of other sulfur compounds (i.e. organisms may be auxotrophs for other sulfur-containing compounds such as cysteine), or use of MTOH in alternate pathways not associated with the salvage of sulfur.

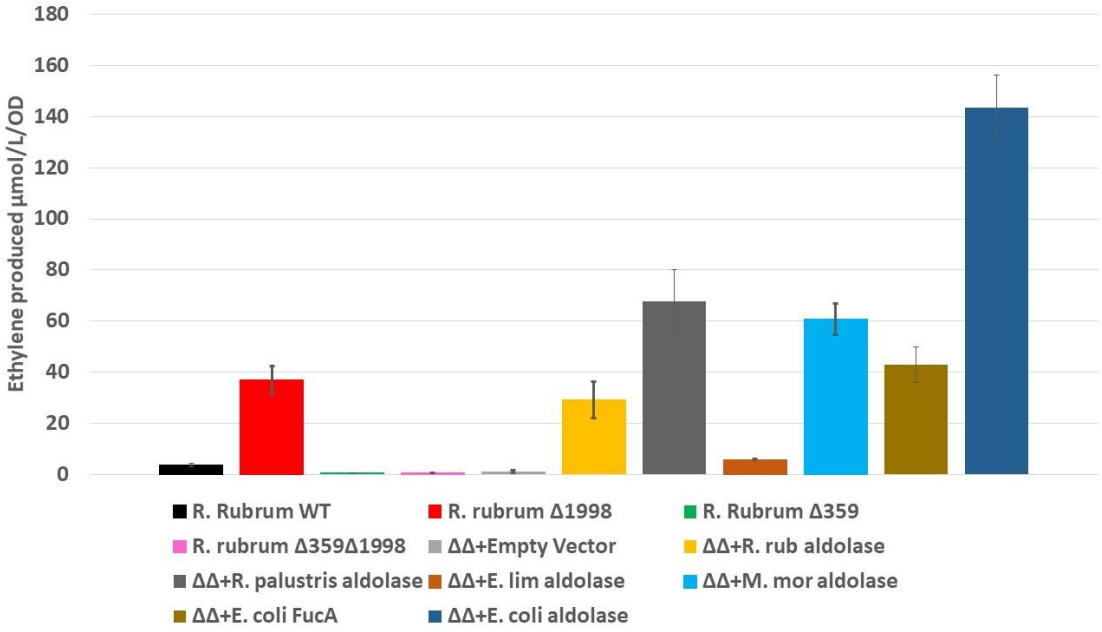

Figure 6: Aldolase gene complementation studies. Maximum titers of ethylene gas produced (µmol/L/OD_{660}) of *R. rubrum* Δ*rlp*Δ*ald2* strain complemented with the *R. rubrum* ATCC 11170 *ald2* gene and various transgenic putative MTRu-1P aldolases from *R. palustris* CGA010, *E. limosum* ATCC 8486, *M. morganii* INSali207, and *E. coli* ATCC 25922. Cultures were grown anaerobically, photoheterotrophically using MMM with 0.25 mM MTA.

Table 1: Growth Phenotypes of strains with putative anaerobic MSPs

	R. rubrum		R. palustris		M. morganii	E. limosum	E. coli ATCC 25922	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic	Aerobic	Anaerobic
1mM SO4	+	+	+	+	N/A	N/A	+	+
1mM Methionine	+	+	+	+	+/-	N/A	N/A	N/A
1mM Cysteine HCl	+	+	+	+	+	+	+	+
0.25mM MTA	+	+	+	+	-	-	-	-
1mM MTOH	-	+	+	+	N/A	-	-	-

Discussion

Based on the results of the complementation experiments, it appears that the complemented aldolase(s) from *R. palustris*, *M. morganii*, and *E. coli* ATCC 25922 can catalyze the conversion of MTRu-1P to DHAP and MTAdh. However, with the exception of *R. rubrum* and *R. palustris*, none of these organisms themselves can utilize either MTA, the starting compound for methionine salvage, or MTOH, the reduced form of MTadh produced by enzymes with MTRu-1P aldolase activity, as a sole sulfur source.

It appears that some organisms encoding putative Ald2 proteins, even ones encoded for in the same genetic region as other methionine salvage genes such as *mtap* and *mtri*, may not contain further downstream methionine salvage genes to fully recycle MTA into usable sulfur compounds. This leads to a few possibilities regarding the presence of these genes.

The genes might act to convert MTA to a non-inhibitory form without fully metabolizing it. Utilizing this partial pathway, this would lead to formation of MTOH as a potential dead-end product. The presence of MTOH has been observed in some wines[17], but it is uncertain whether this compound is formed enzymatically through wine-associated bacterial species or through non-enzymatic degradation pathways[17]. Further supporting this observation, when the *E. coli* ATCC 25922 strain is fed with radiolabeled [^{14}C]MTA, [^{14}C]MTOH is observed to be excreted into the spent media (J.A.N. unpublished results). However, this observation does not provide a metabolic rationale for the formation of MTOH beyond elimination of MTA-induced inhibition of cellular growth, which hypothetically could be done by just MTA phosphorylase (MTAP) or MTA nucleosidase (MtnN) (see Figure 1).

Another possibility is that the *mtap-mtri-ald2* operon may act upon a different substrate in the bacterial cells for a purpose other than methionine salvage. One such potential pathway is the metabolism 5'-deoxyadenosine, and this is analyzed in Chapter IV.

Chapter IV: Characterization of a novel 5'-deoxyadenosine salvage pathway in *R. rubrum*

Introduction

As described earlier, S-adenosylmethionine (SAM) is an important component for many metabolic reactions in cells. Reactions involving SAM can be categorized predominately in three ways based on the metabolic waste product that is produced[18], and a schematic of these reactions and their byproducts is presented in Figure 7.

Some reactions, including polyamine synthesis and homoserine lactone production, result in MTA as a byproduct[1,18] (Figure 7, blue pathway). Notably, there are many known salvage pathways for MTA, as discussed in Chapters I and II. These pathways lead to the salvage of the sulfur from MTA and some if not all of the carbon of MTA[1-5,12].

Another set of reactions, used for methylation of DNA, RNA, and proteins leads to a byproduct called S-adenosylhomocysteine (SAH)[18] (Figure 7, green pathway). This compound has two known salvage pathways. In most eukaryotes and some prokaryotes, SAH hydrolase cleaves SAH into homocysteine and adenosine[20]. In other prokaryotes, adenine is cleaved from SAH by an SAH nucleosidase to form S-ribosylhomocysteine[19,20]. This molecule is cleaved by S-ribosylhomocysteinase to from homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor to the quorum sensing molecule autoinducer-2[20]. In both cases, the sulfur can be recycled by converting homocysteine to methionine via methionine synthase[19]. Furthermore, the rest of the carbon from SAH is metabolized into either adenosine or 4,5-dihydroxy-2,3-pentanedione, which have known cellular uses.

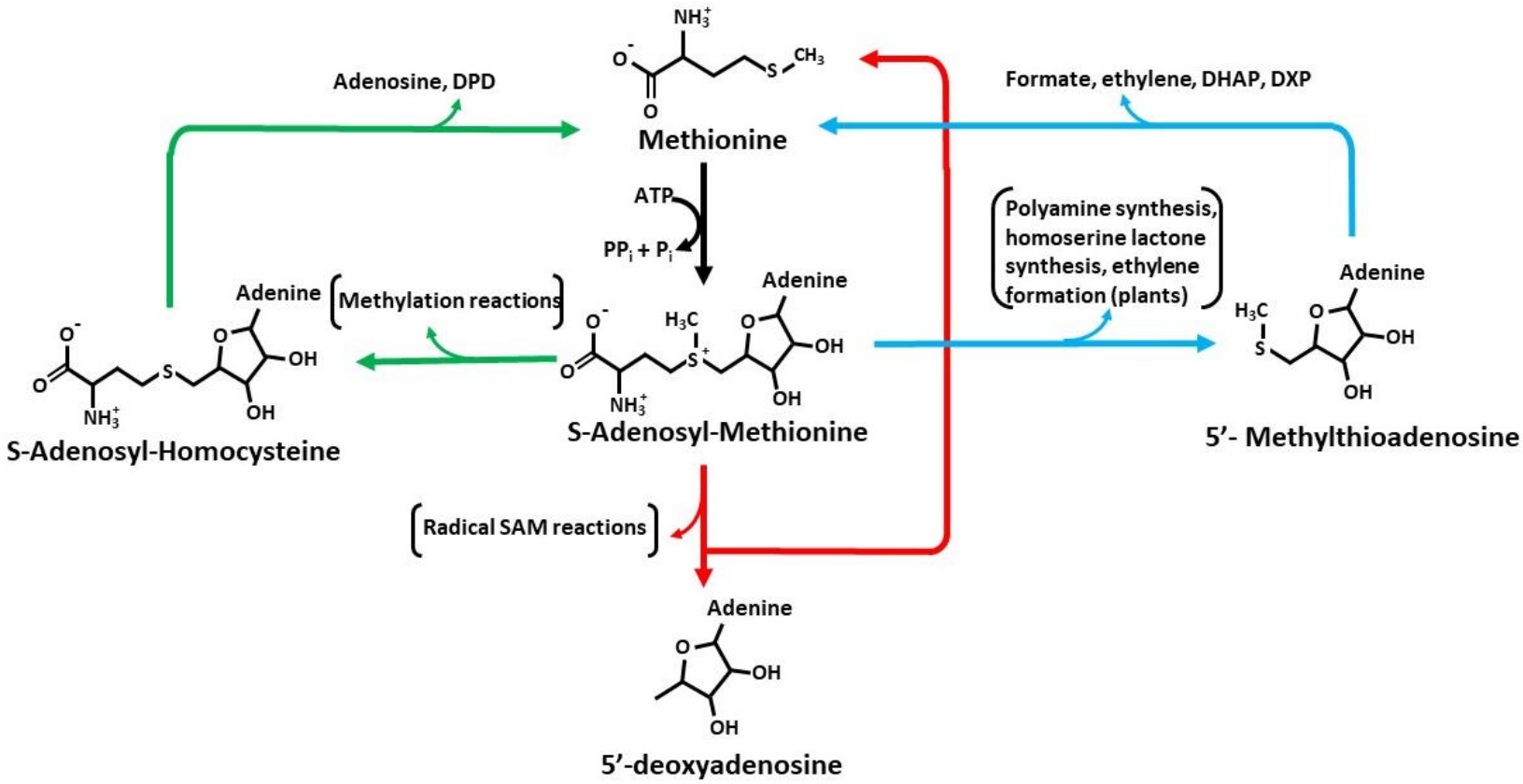

Figure 7: Common reactions involving S-adenosylmethionine. In green, methylation of various substates using SAM-dependent methylases, resulting in the formation of S-adenosylhomocysteine as a byproduct. In red, Radical SAM reactions, resulting in the formation of 5'-deoxyadenosine. In blue, reactions such as polyamine synthesis, homoserine lactone synthesis, and ethylene formation in plants that leads to formation of methylthioadenosine. **Abbreviations:** DPD, 5-dihydroxy-2,3-pentanedione; DHAP, dihydroxyacetone phosphate; DXP, 1-deoxyxylulose-5-phosphate; ATP, adenosine triphosphate.

The third type of reactions are catalyzed by radical-SAM enzymes. Radical-SAM enzymes are a diverse family of enzymes used for creation of cofactors, small metabolites, and some methylation reactions[22-24] (Figure 7, red pathway). While they are used in metabolically diverse activities, these enzymes generally share a CX_3CX_2C motif that is used to coordinate an iron-sulfur cluster[22]. The iron sulfur cluster is used in the reductive cleavage of SAM to form a 5'-deoxyadenosyl radical (5dAdo•)[24]. This radical is a strong oxidant which can be used to abstract a hydrogen from a target compound[24]. While the targets of radical-SAM enzymes vary, the abstraction of a proton results in the formation of 5'-deoxyadenosine (5dAdo)[18].

Interestingly, while salvage pathways for SAH and MTA are well-characterized, there are no well characterized metabolic salvage pathways for 5dAdo. Previous studies have shown that 5'-deoxyadenosine can be acted upon by MTAP in mammalian cancer cell lines to form 5'-deoxyribose-1-phosphate, presumably part of purine salvage[21]. This product was then detected to be acted upon by a purine nucleoside phosphorylase to form 5'-deoxyinosine[21]. Studies in the archaeon *Methanocaldococcus jannaschii* demonstrated that 5'-deoxyadenosine could be converted to 5'-deoxyinosine using a 5′-Deoxyadenosine deaminase[31]. The authors hypothesized this product could be acted upon by two native enzymes homologous to MTAP and MTRI, resulting in the formation of 5'-deoxyribulose-1-phosphate[31]. They postulated that this compound may somehow form 6-deoxy-5-keto-fructose-1-phosphate, a precursor for some archaeal aromatic amino acid biosynthesis pathways, albeit by unknown mechanisms[31]. However, no studies have definitively described a way that 5'-deoxyadenosine could be recycled into a useful cell product as part of carbon salvage.

Based on these findings and the chemical similarity of 5dAdo and MTA, it was hypothesized that native methionine salvage enzymes in *R. rubrum* may also be utilized in

carbon salvage of 5dAdo. This hypothesized pathway is presented in Figure 8. First, 5dAdo is likely converted to 5-deoxyribulose-1-phosphate (5dRu-1P) by sequential action of MTAP and MTRI, Then, depending on whether the *rlp* or *ald2* gene products acted upon 5dRu-1P, two different products could be obtained. If acted upon by RLP in a manner similar to that of methionine salvage[3], 5-deoxyribulose-1-phosphate would likely be converted into 1-deoxyxylulose-5-phosphate (DXP), which is used in isoprenoid biosynthesis[22]. If acted upon by Ald2 in a manner similar to that of methionine salvage[12], 5-deoxyribulose-1-phosphate would be converted into dihydroxyacetone phosphate (DHAP) and acetaldehyde. Both of these compounds have known mechanisms to enter central carbon metabolism. Thus, either pathway could potentially be used to salvage the carbon from 5dAdo.

In order to determine the potential function of methionine salvage genes and enzymes in 5dAdo metabolism, *R. rubrum* and *R. palustris* knockout strains of methionine salvage genes were grown and tested for the excretion of 5dAdo. Note that *R. palustris* has two RLPs, labelled RLP1 and RLP2. RLP1 is a member of the Deep-YkrW clade, similar to *R.* rubrum RLP[3], whereas RLP2 is from the form-IV Photo clade of RLPs[3]. As 5dAdo can be inhibitory to the growth of cells[18,19], it is likely to be excreted when in excess (J.A.N. unpublished observations). Hypothetically, knocking out genes involved with 5dAdo salvage could lead to the accumulation and thus excretion of 5dAdo.

Elevation in excreted 5dAdo levels was observed in *R. palustris* and *R. rubrum* Δ*mtap* and Δ*mtri* knockout strains, suggesting that these enzymes function in the salvage of 5dAdo in these organisms, especially under anaerobic conditions. In order to further verify their function and test the potential activity of subsequent salvage enzymes, the metabolic pathway was reconstituted enzymatically using [^{3}H]5'-deoxyadenosine and purified *R. rubrum* MTAP, MTRI,

RLP, and *R. palustris* RLP1 and RLP2 to allow for detection of intermediate metabolites. These assays confirmed the ability of MTAP and MTRI to act on 5'-deoxy substrates, further supporting their role in a putative 5dAdo salvage pathway.

Detailed materials and methods for Chapter IV are given in Chapter V, Section IV.

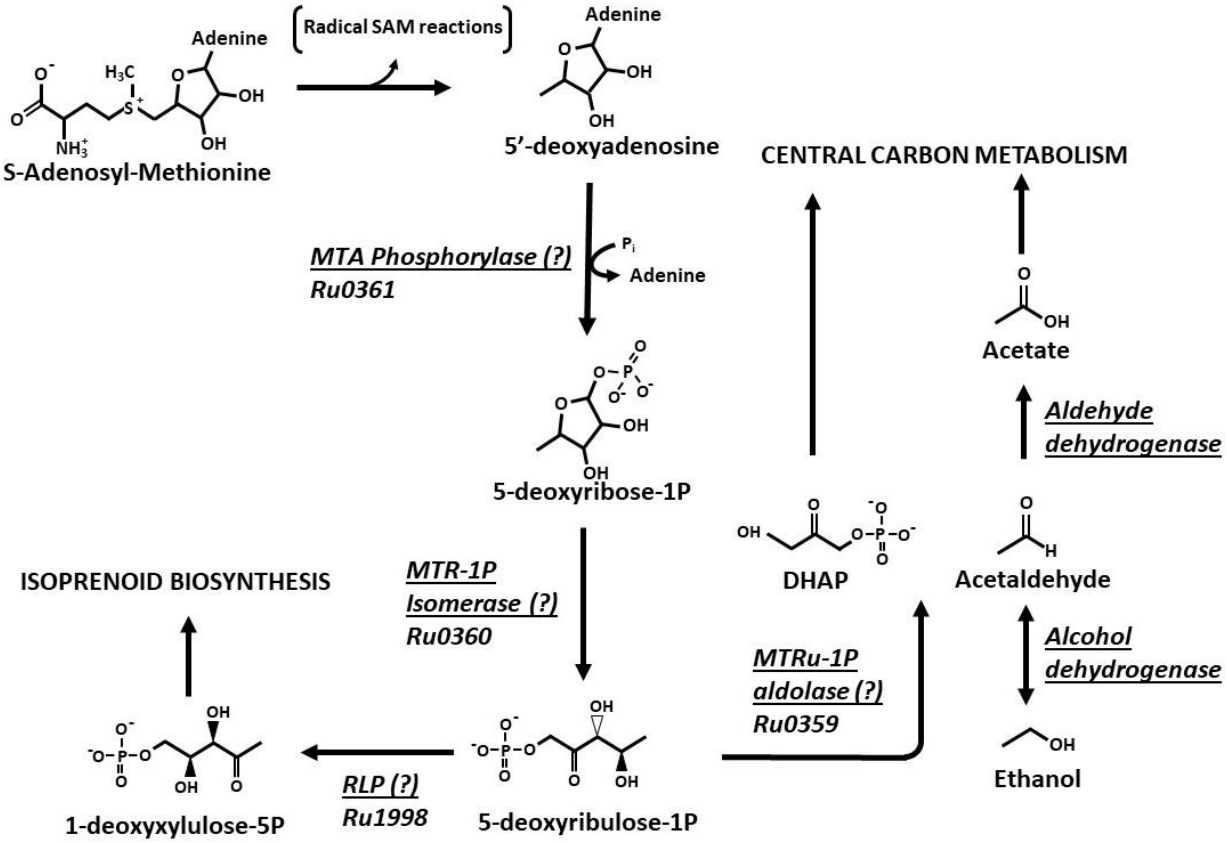

Figure 8: Hypothetical 5'-deoxyadenosine salvage pathways. Schematic of how known *R. rubrum* methionine salvage enzyme may also be involved in 5dAdo recycling. **Abbreviations:** DHAP, dihydroxyacetone phosphate; ATP, adenosine triphosphate

Results

For the quantification of 5dAdo excretion, the maximal concentrations of accumulated 5dAdo are presented in Table 2, and some selected time courses are presented in Figure 9.

For *R. palustris* grown under anaerobic conditions, 5dAdo was excreted into the spent media by all strains. However, deletion of the *mtap* gene or *mtri* gene led to a roughly three-fold increase in the amount of 5dAdo excreted, suggesting disruption of an internal 5'-deoxyadenosine salvage pathway. Interestingly, deletion of the *rlp2* gene led to a two-fold increase in 5dAdo excretion, suggesting that it may function in the salvage of 5dAdo under anaerobic conditions as well. While the various *R. rubrum rlp, R. palustris rlp1*, and *ald2* deletion strains did not have enhanced 5dAdo excretion when compared to WT, the function of these genes in 5dAdo salvage cannot be ruled out using this methodology.

Under aerobic conditions, the *R. palustris* WT strain excreted 5dAdo into the spent media at approximately 25-fold lower levels than under anaerobic conditions, consistent with the fact that radical SAM enzymes are oxygen sensitive and primarily function under anaerobic conditions. Like for anaerobic conditions, under aerobic conditions Δ*mtap* and Δ*mtri* had 3.5- and 2-fold increases in excreted 5dAdo than WT respectively, suggesting that they still may be used to salvage 5dAdo under aerobic conditions. Δ*rlp*2 did not lead to enhanced 5dAdo excretion over WT under aerobic conditions, unlike when it was grown under anaerobic conditions. The *R. rubrum rlp, R. palustris rlp1*, and *ald2* deletion strains similarly did not have enhanced 5dAdo excretion when compared to WT.

Table 2: Maximum excreted 5'-deoxyadenosine (μM)

Aerobic	R palustris	R rubrum
Δmtap	6.90 ± 0.45	0.64 ± 0.03
Δmtri	4.47 ± 0.12	0.64 ± 0.04
Δald2	N/A	0.43 ± 0.13
Δrlp1	1.44 ± 0.22	0.74 ± 0.7
Δrlp2	2.14 ± 0.09	N/A
Δrlp1Δald2	2.17 ± 0.47	0.75 ± 0.07
WT	2.03 ± 0.21	0.15 ± 0.01
Δmtap + mtap	1.25 ± 0.16	0.46 ± 0.10
Δmtri + mtri	0.67 ± 0.40	0.25 ± 0.03
Δald2 + ald2	N/A	0.21 ± 0.01
Δrlp1Δald2 + ald2	1.83 ± 0.16	0.52 ± 0.02
Anaerobic	R palustris	R rubrum
Δmtap	172.43 ± 3.82	89.98 ± 2.96
Δmtri	131.70 ± 8.14	0.66 ± 0.12
Δald2	N/A	0.19 ± 0.06
Δrlp1	54.62 ± 3.49	0.31 ± 0.03
Δrlp2	91.60 ± 3.49	N/A
Δrlp1Δald2	55.38 ± 3.44	0.21 ± 0.05
WT	46.82 ± 5.95	0.22 ± 0.01
Δmtap + mtap	55.56 ± 4.27	0.14 ± 0.05
Δmtri + mtri	24.69 ± 1.10	0.14 ± 0.02
Δald2 + ald2	N/A	0.18 ± 0.06
Δrlp1Δald2 + ald2	55.33 ± 7.07	0.13 ± 0.01

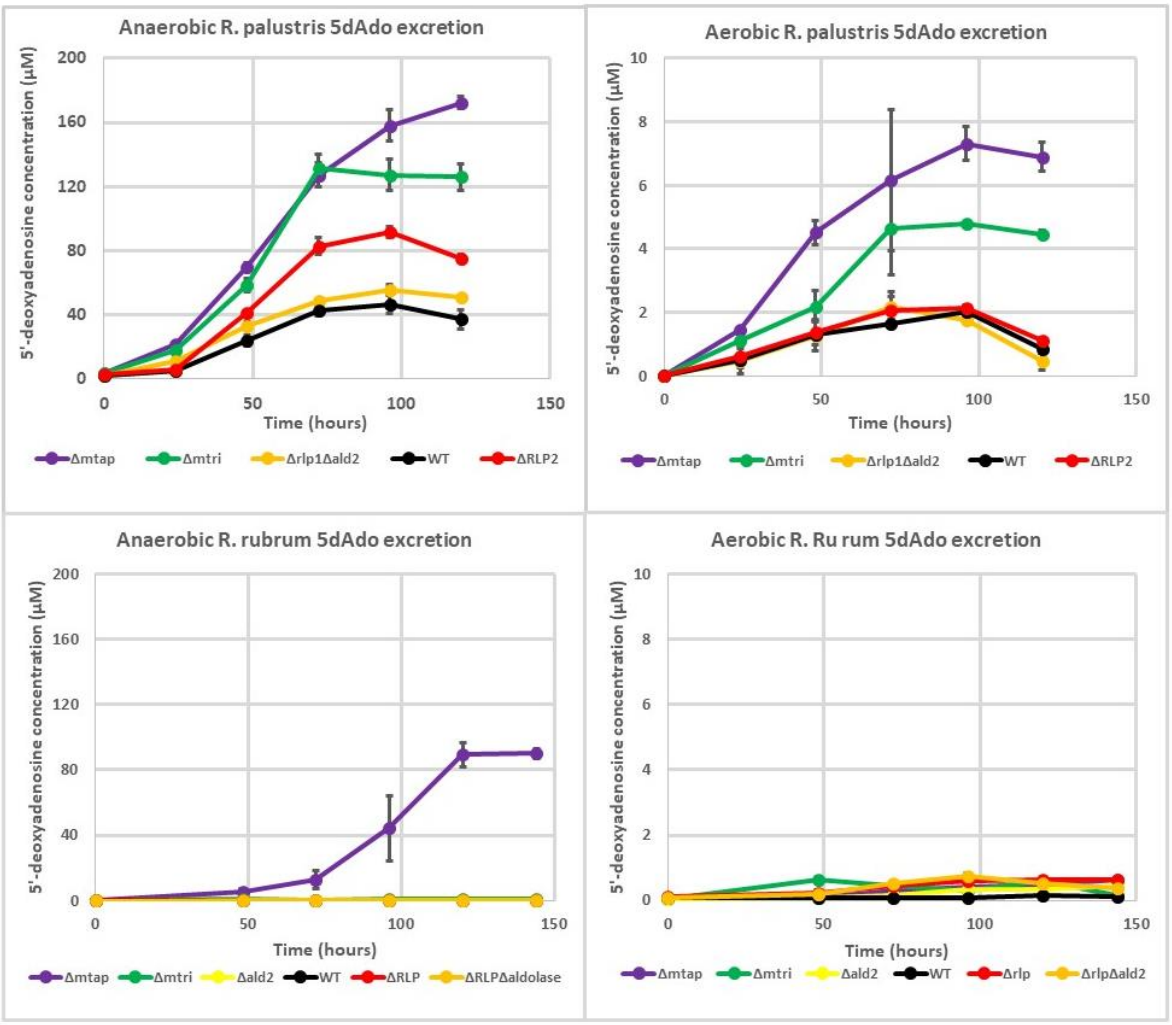

Figure 9: 5'-deoxyadenosine excretion. Concentrations of accumulated 5'-deoxyadenosine in the spent media of various *R. rubrum* and *R. palustris* knockout strains of MSP genes. Growth experiments were performed under aerobic and anaerobic conditions.

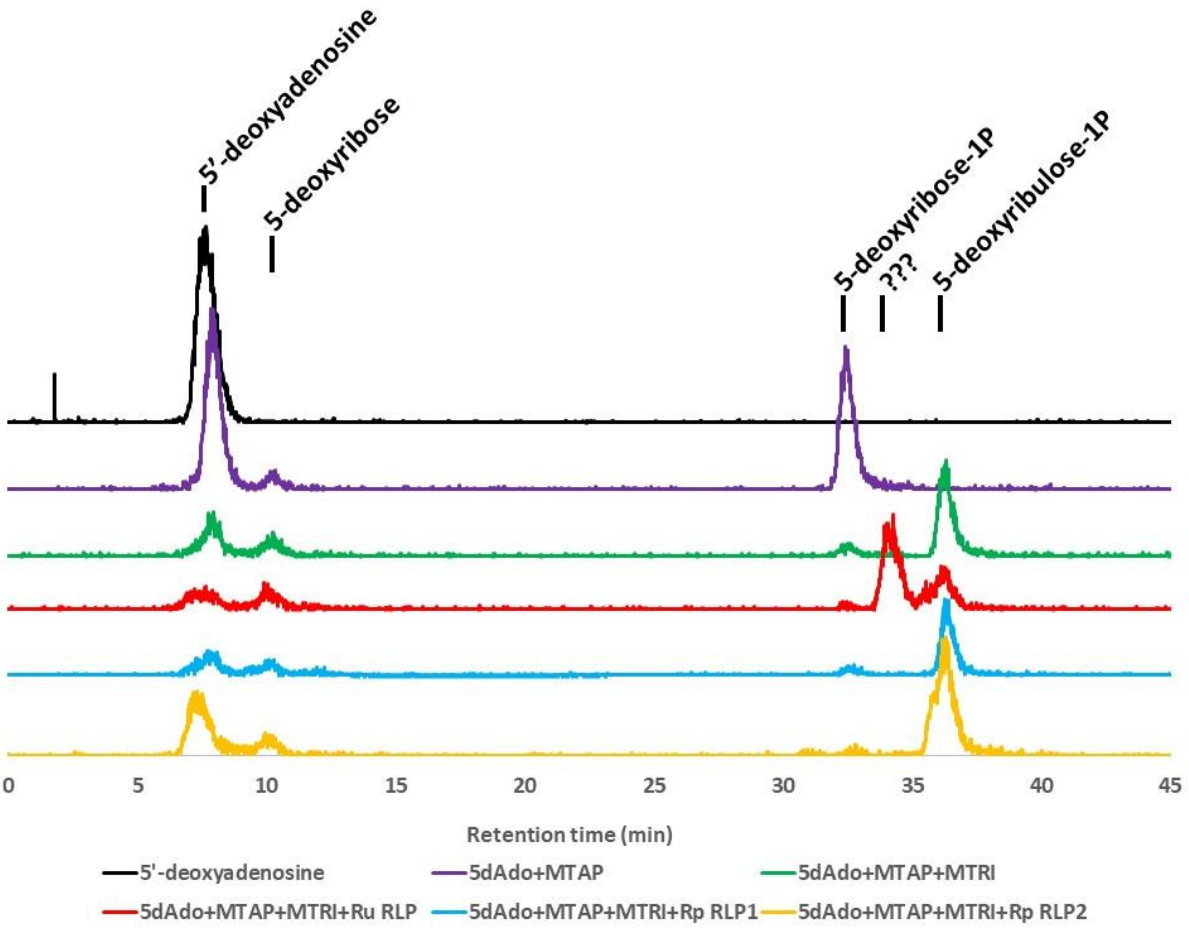

Figure 10: 5'-deoxyadenosine salvage pathway metabolite analysis. Conversion of [^{3}H]5'-deoxyadenosine sequentially by purified recombinant MTAP, MTRI, and either *R. rubrum* RLP, *R. palustris* RLP1, or *R. palustris* RLP2. Separation of peaks performed by a SeQuant zic-pHILIC column connected to a Shimadzu Prominence HPLC. Labelled peaks are the hypothetical products according to the putative 5'-deoxyadenosine salvage pathway postulated in *R. rubrum*.

Under anaerobic conditions in *R. rubrum*, most strains excreted negligible levels of 5dAdo. The only exception was the Δ*mtap* strain, which excreted 5dAdo at levels at roughly a 400-fold increase over WT, suggesting that deletion of *mtap* disrupted 5dAdo salvage in *R. rubrum*. Deletion of *mtri* led to approximately 2-fold increases in 5dAdo when compared to WT as well. Note that while no other strains (Δ*rlp*, Δ*ald2*, or Δ*rlp*Δ*ald2*) had enhanced 5dAdo production, their function in 5dAdo salvage cannot be ruled out by this methodology.

Under aerobic conditions in *R. rubrum*, all strains produced negligible levels of 5dAdo.

The results of the enzymatic reconstitution are presented in Figure 10. 5dAdo had a retention time of ~7.4 minutes. Addition of purified MTAP to 5dAdo led to roughly 50% conversion into a new peak with a retention time of ~32.5 minutes, presumably 5-deoxyribose-1-phosphate. Further addition of MTRI to the reaction resulted in the appearance of a third peak with a retention time of 36.1 minutes, presumably 5-deoxyribulose-1-phosphate.

Addition of *R. rubrum* RLP resulted in the formation of a new peak with a retention time of ~34.1 minutes. Furthermore, there appeared to be a potential peak with a retention time of ~35.5 minutes that partially overlapped with 5dRu-1P. A comparison of the raw data and data with 20-point averaging (0.15 second moving window) are shown in Figure 11, which allows for improved signal-to-noise in distinguishing the peaks. Presumably, one of the compounds formed by *R. rubrum* RLP would be DXP. However, when DXP reductoisomerase (DRI) was added to the reaction described above, no conversion of either peak was observed (Figure 12).

Furthermore, when the oxidation of NADPH by DRI was measured spectrophotometrically, there was no oxidation of NADPH in the presence of 5dAdo with MTAP, MTRI, *R. rubrum* RLP, and DRI. As a positive control to make sure that 5dAdo derivatives were

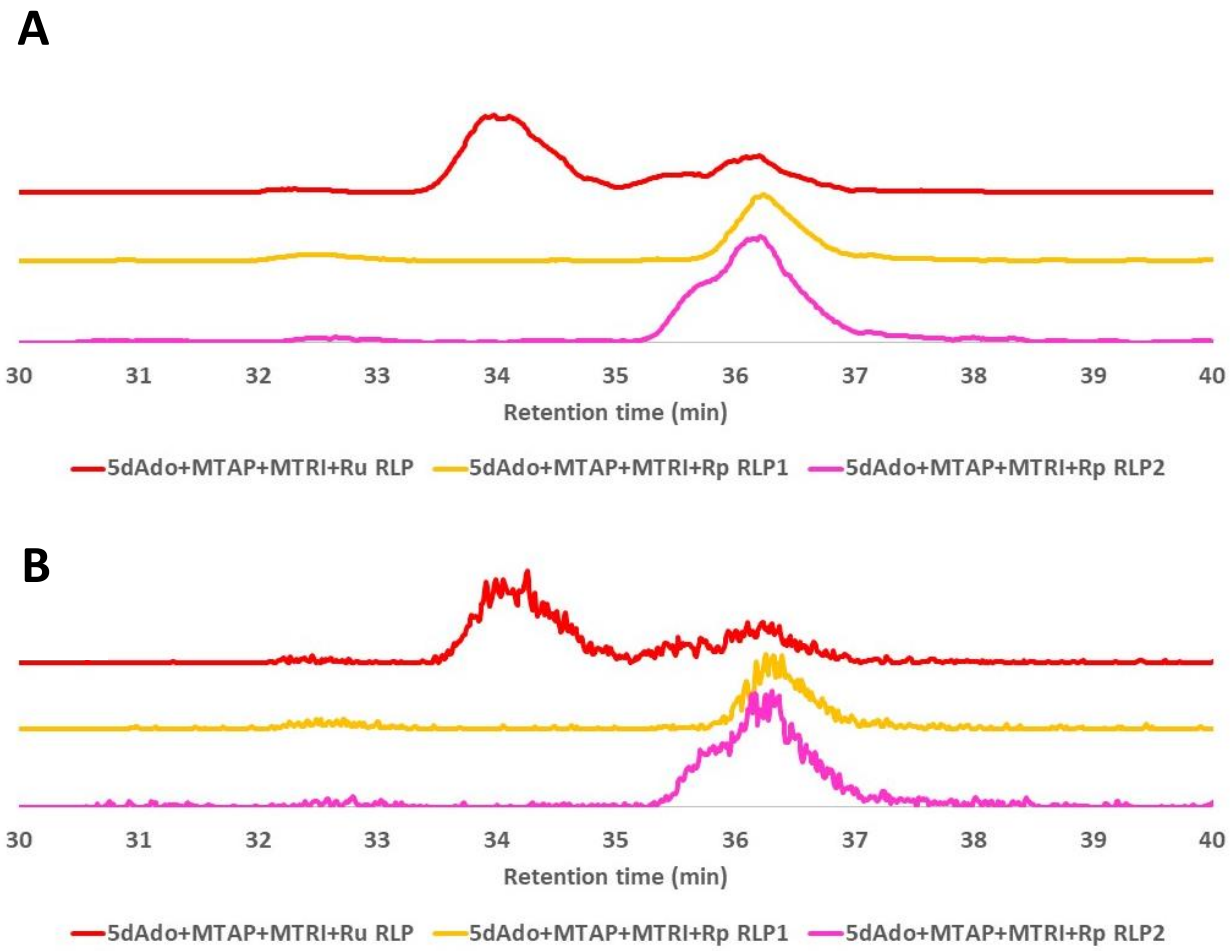

Figure 11: Separation of 5-deoxy-pentosephosphate metabolites. (A) separation of phosphorylated [^{3}H]5'-deoxyadenosine derivatives with 20-point (0.15 second) data averaging. Derivatives were synthesized by purified recombinant MTAP, MTRI, with either *R. rubrum* RLP, *R. palustris* RLP1, or *R. palustris* RLP2. (B) original unsmoothed data

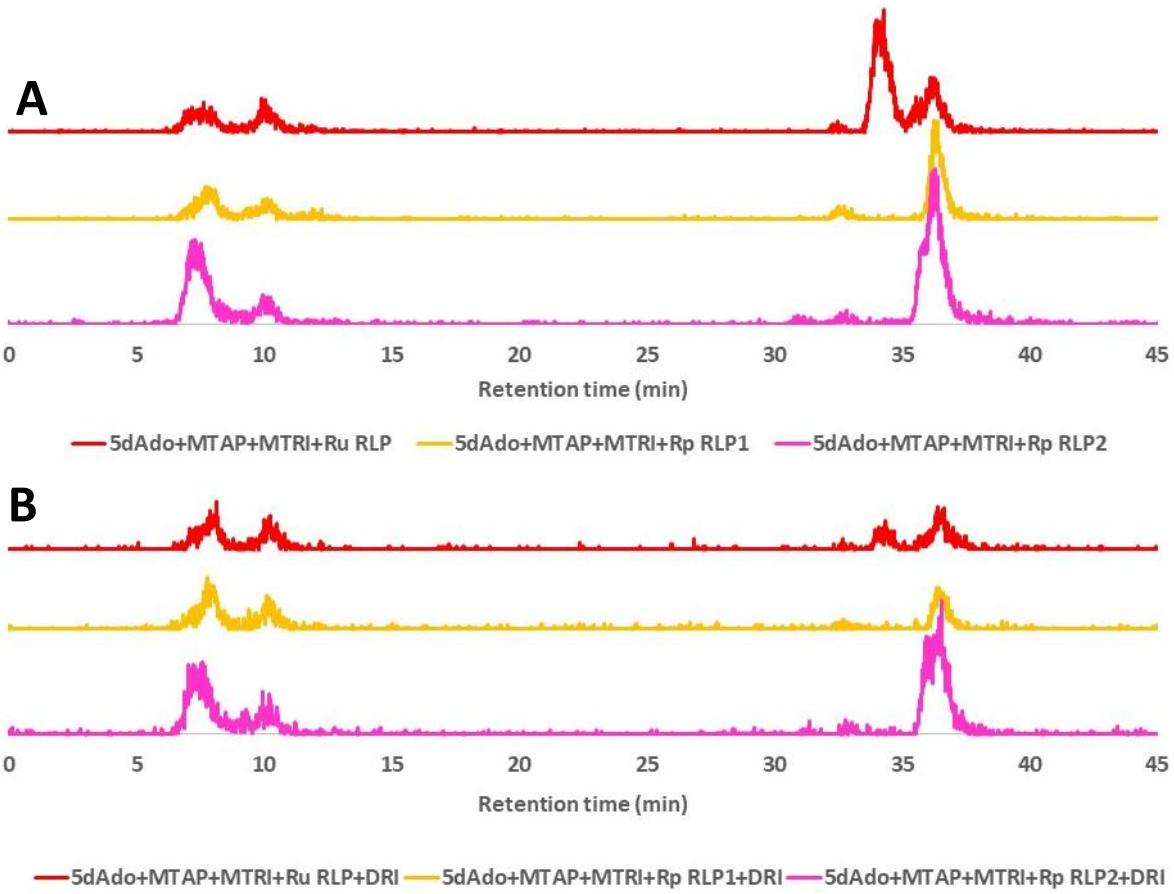

Figure 12: Test for DXP with DXP reductoisomerase. (A) conversion of [^{3}H]5'-deoxyadenosine sequentially by purified recombinant MTAP, MTRI, and either *R. rubrum* RLP, *R. palustris* RLP1, or *R. palustris* RLP2. (B) conversion of [^{3}H]5'-deoxyadenosine sequentially by purified recombinant MTAP, MTRI, DXP reductoisomerase (DRI), and either *R. rubrum* RLP, *R. palustris* RLP1, or *R. palustris* RLP2. The addition of DRI resulted in no new peak formation, implying no 1-deoxyxylulose-5-phosphate was formed by the action of MTAP, MTRI, and any RLP on [^{3}H]5'-deoxyadenosine.

not inhibiting any enzymes, MTA and the cupin enzyme were added to the mixture, which resulted in the creation of DXP as part of methionine salvage (data not shown). This led to the oxidation of NADPH, showing that the system was valid, but that no 5dAdo derivative had been converted to DXP.

Addition of *R. palustris* RLP1 had no noticeable effect on the enzymatic system, suggesting that it cannot function on 5dAdo derivatives.

Addition of *R. palustris* RLP2 resulted in a potential peak (see Figure 11) overlapping with 5dR-1P, similar to that seen with the *R. rubrum* RLP. The approximate retention time was ~35.8 minutes. However, it is uncertain if this peak is the same species as that formed by the *R. rubrum* RLP.

Discussion

Overall, the function of MTAP and MTRI in a putative 5'-deoxyadenosine salvage pathway in *R. rubrum* and *R. palustris* was supported by the *in vivo* quantification of 5dAdo excretion and *in vitro* reconstitution of the putative pathway. Deletion of *mtap* in *R. rubrum* and *R. palustris*, and deletion of *mtri* in *R. palustris* led to accumulation of 5dAdo in the spent media under anaerobic conditions. This suggests that natural 5dAdo metabolic pathways were disrupted. Furthermore, it was shown that purified MTAP and MTRI from *R. rubrum* could act sequentially on 5dAdo *in vitro*, presumably forming 5-deoxyribose-1-phosphate and 5-deoxyribulose-1-phosphate respectively.

The function of *R. rubrum* RLP in 5dAdo metabolism was also partially supported in this experiment. Purified RLP was able to act on the product of MTRI, presumably 5-deoxyribulose-1-phosphate. The logical product of this reaction, based on known RLP function[33] would be

DXP. However, when DRI, which is known to reduce DXP[22], was added to the RLP reaction product, there was no conversion of the unknown peak. Further testing showed that DRI could not reduce NADPH in the presence of the RLP reaction, giving more evidence that the unknown peak was not DXP. Thus, it appears that *R. rubrum* RLP can act upon 5-deoxy species, but its products are unknown. Further analysis of the product of RLP may be obtained by tandem HPLC and mass spectrometry (HPLC/MS).

With regard to *R. palustris*, RLP1 showed no activity *in vivo* upon 5-deoxy species. However, purified RLP2 showed potential activity for the conversion of 5-deoxyribulose-1-phosphate to an unknown peak overlapping with 5-deoxyribulose-1-phosphate. The function of RLP2 in a 5dAdo salvage mechanism was also supported by an *in vivo* buildup of 5dAdo in the RLP2 knockout under anaerobic conditions. Further analysis via HPLC/MS may shed light on the potential products, if any, of RLP2 upon 5-deoxy species.

If RLP2 does have function in 5dAdo salvage, this would be the first definitive mechanism for form-IV Photo RLPs. The form-IV Photo RLP from *Chlorobium tepidum* has been implicated in thiosulfate oxidation, but its exact function remains unknown[3]. It is also interesting that the RLP1, which is a Form-IV Deep YkrW RLP and has shared methionine salvage function with *R. rubrum*'s RLP[3], is unable to catalyze any reaction with 5-deoxyribulose-1-phosphate while *R. rubrum*'s RLP appears to have some function.

Further characterization is also required for the function of Ald2 in 5dAdo metabolism. No support for its function was found in this study, but a similar *in vitro* enzymatic assay would be able to point to its function in a 5dAdo salvage pathway. Such a function would lead to wider implications on carbon salvage, as over 200 organisms encode enzymes homologous to MTAP, MTRI, and Ald2[12]. Notably, 83% of these organisms are obligate or facultative anaerobes[12].

Radical SAM reactions involve the use of an iron-sulfur cluster[24], which are heavily susceptible to oxidation. This could explain why these genes are more prevalent in anaerobic organisms, as Radical SAM reactions would be more stable under anaerobic conditions.

Further enzymatic assays combined with definitive identification of 5'-deoxy species through mass spectrometry will be able to confirm the identity of discovered chemical intermediates in the hypothetical 5dAdo salvage pathway. Furthermore, cellular feedings of *R. rubrum* knockout strains with tritiated 5dAdo could be used to prove the function of this pathway *in vivo*.

Chapter V: Detailed methods

Section I: Chapter I methods

Construction of the Δ*mtap* and Δ*mtri* knockout strains

Cloning techniques

All genetic manipulations done in the course of this thesis utilized DNA olignonucleotide primers ordered from Sigma-Aldrich and enzymes obtained from New England Biolabs (NEB), unless otherwise stated. All primer sequences can be found in Appendix I. All enzymatic reactions for cloning were undertaken according to the manufacturer's protocols. Purification of nucleic acids after PCR or gel electrophoresis was done using commercially available purification kits from QIAGEN, including QIAGEN PCR-clean-up, QIAGEN DNEasy kit, and QIAGEN gel-purification kits. All gel-electrophoresis of DNA fragments was performed using 0.8% agarose gels in 0.5x Tris Acetate EDTA buffer and using commercial 1kb-DNA-ladders from NEB. Agarose gels were run at 120V.

Construction of pk18-mobsacB-Gm

The vector backbone for construction of knockout plasmid was pk18-mobsacB-Gm. This was constructed from pk18-mobsacB by insertion of a gentamycin-resistance cassette into an endogenous NheI restriction site. Primers GenF and GenR (see Appendix I) were used to PCR amplify the gentamycin-resistance cassette from pUC1318-gm. Each primer also contained a restriction site for NheI. The resulting fragment was enzymatically digested using NheI and purified using a QIAGEN PCR-cleanup kit. The pk18-mobsacB plasmid was also enzymatically digested with NheI and treated with Antarctic phosphatase to reduce plasmid recircularization

during ligation, and then purified using a QIAGEN PCR-cleanup kit. The fragment and vector were then ligated together using T4 DNA ligase, and the ligation mixture was used to transform *E. coli* Top10 cells to allow for amplification and purification of the vector, as described below.

Transformation of plasmids into *E. coli* strains

Vectors were used to transform various chemically competent *E. coli* strains. Either 1 μL of purified plasmid or 10 μL of a ligation product were added to a 100 μL aliquot of chemically competent *E. coli* cells, and the mixture was incubated on ice for 20 minutes. The aliquot was then heat-shocked at 42C for 1 minute, and subsequently incubated on ice for 5 minutes. Then, 0.5 mL of SOC media (SOC, Appendix II) was added to the mixture and the cells were placed into a 37C shaking incubator for one hour. The cells were then spread-plated onto 16% agar-lysogeny broth (LB, Appendix II) plates with the appropriate concentration of antibiotics (Appendix III). Individual colonies were picked and grown in 5 mL of LB, and the plasmids were extracted using a QIAGEN Miniprep kit. Plasmid sequences were verified by sequencing through the Plant Microbe Genomic Facility (PMGF) at Ohio State.

Construction of the truncated gene fragment

A schematic for the construction of the gene fragment is provided in Figure 13.

To construct the knockout plasmid for *mtap*, primers were designed that would amplify two fragments, designated the MTAP-F1 and MTAP-F2 fragments. The MTAP-F1 fragment consisted of approximately 1000 base pairs upstream of the gene of interest and the first 45 bases of the gene of interest. The forward primer, 361KF, contained a restriction enzyme site for XhoI. The reverse primer, 361KR, contained a restriction site for XbaI. The F2 fragment consisted of approximately the last 45 bases of the gene of interest, and approximately 1000 base pairs

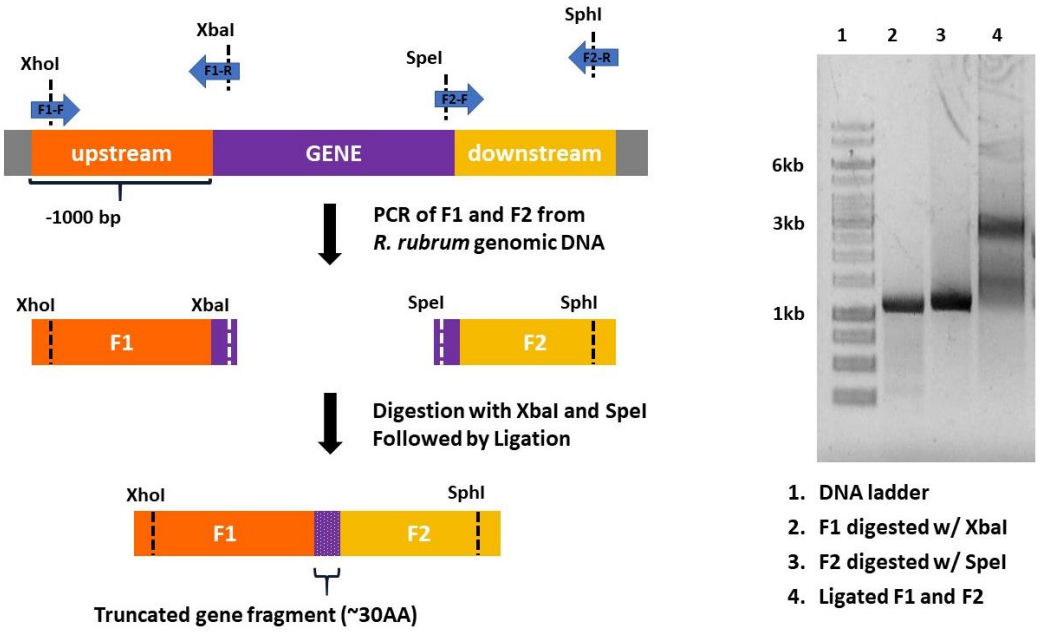

Figure 13: Construction of DNA fragments for gene deletion. Schematic of the construction of a gene knockout fragment for use in homologous recombination-mediated gene knockouts.

downstream of the gene of interest. The forward primer, 361SF, contained a restriction site for SpeI. The reverse primer, 361KR, contained a restriction site for SphI. These two fragments were amplified using PCR using Phusion DNA polymerase according to the manufacturer's protocol, and purified using a commercial QIAGEN PCR-cleanup kit.

The MTAP-F1 and MTAP-F2 fragments were then enzymatically digested using XbaI and SpeI, respectively, and purified using the Qiagen PCR-cleanup kit. Equal amounts of the two fragments were combined together and the fragments were ligated together using T4 ligase. After ligation, the mixture was re-digested with XbaI and SpeI to eliminate undesired products in which two MTAP-F1 or two MTAP-F2 fragments ligated together. However, due to the hybrid XbaI-SpeI site constructed by the desired ligation of MTAP-1F to MTAP-F2, this product remained in-tact and was purified from the mixture by 0.8% agarose gel purification using the QIAGEN gel-purification kit. This resulting MTAP-F12 fragment consisted of an in-frame, 90 base pair (30 amino acid) truncated *mtap* gene fragment flanked on either side by ~1000 base pairs of the natural upstream and downstream regions for the *mtap* gene (Figure. 13) . This fragment was re-amplified via PCR using the 361KF and 361KR primers to increase the available quantity.

Construction of the knockout vector

To complete the knockout plasmid, the MTAP-F12 fragment and pk18-mobsacB-Gm plasmid were separately digested using XhoI and SpeI restriction enzymes. The plasmid was also treated with Antarctic phosphatase. Digested DNA fragments were purified using a QIAGEN PCR-cleanup kit. The purified pk18-mobsacB-Gm and MTAP-F12 fragment were then ligated together using T4 DNA ligase into the final pk18-mobsacB-Gm-MTAP knockout plasmid. This ligation was transformed into *E. coli* Top10 cells via the protocol above.

The pk18-mobsacB-Gm-MTRI knockout plasmid had previously been constructed by other members of the Tabita lab (J.A.N. unpublished work).

Mating of the knockout vector into *R. rubrum* strains

To delete the *mtap* and *mtri* genes in *R. rubrum*, the pkK18-mobsacB-Gm-MTAP and pk18-mobsacB-MTRI plasmids were first transformed into chemically competent SM10 *E. coli* cells using the protocol described above. SM10 cells that contained the plasmid were selected for their ability to grow on 16% agar-LB plates containing gentamycin. These cells were then used to transfer the vectors into *R. rubrum* via conjugative transfer using biparental mating.

To conduct the biparental mating, *E. coli* SM10 cells containing the relevant plasmid were grown in 5 mL of LB in a 37C shaking incubator. *R. rubrum* wild-type (WT) cells were grown in 20 mL of CSOC (CSOC, Appendix II) in a 30C shaking incubator. *R. rubrum* strain I19* [ΔRuBisCO/NifA(M173V)] was also grown in a similar manner. 5 mL of each strain was pelleted into a 1.5 mL centrifuge tube at 16000 xG for 1 minute, and resuspended with 1 mL PYE media to remove antibiotics. This wash step was repeated two more times. After the final wash step, the *E. coli* cells with the vector and corresponding *R. rubrum* strain(s) (WT for *mtap*, WT and I19* for *mtri*) were then combined into a single 1.5 mL centrifuge tube, centrifuged, and resuspended in 50 μL of PYE. This mixture was then used to form a mating spot on a 16% agar-PYE plate without antibiotic selection, which was allowed to incubate at 30C for 48 hours.

In order to isolate *R. rubrum* cells that contained the knockout plasmid, cell mass from the mating spot was used in a four-phase streak to inoculate a 16% agar-PYE plate that contained antibiotics specific for *R. rubrum* (streptomycin) and the given knockout vector (gentamycin).

Individual colonies were then streaked onto nonselective 16% agar-PYE plates in order to obtain the first and second recombinants, as discussed below.

Selection for first and second recombinants

A schematic for the selection of first and second recombinants is shown in Figure 14.

R. rubrum cells resistant to gentamycin presumably had undergone homologous recombination with the genome to insert the plasmid into the chromosome. These cells, designated the first recombinants, were selected for gentamycin resistance and sucrose sensitivity, conferred by the *sacB* gene. These cells were then repeatedly grown and sub-cultured using non-selective liquid PYE media at 30C in a shaking incubator to allow time for the second recombination event to occur.

In the second recombination event, the homologous regions between the native genome and integrated vector (see schematic in Figure 14) recombine again with two possibilities, excising the vector backbone and truncated gene fragment (reversion to wild-type), or excising the vector backbone and native gene, leaving only the truncated gene fragment in the strain (conversion to the knockout strain). Second recombinants were selected by identifying cells that could grow on sucrose (i.e. *sacB* gene had been excised) and that were sensitive to gentamycin. To differentiate between wild-type and knockout strains, the *mtap* or *mtri* genes were amplified using PCR, and fragment sizes were determined via gel-electrophoresis.

Putative knockout strains with the correct fragment length were verified by genomic DNA extraction using a QIAGEN DNEasy Tissue kit, and subsequent gene sequencing by the OSU Plant Microbe Genomics Facility. The verified strains were designated Δ*mtap*, Δ*mtri*, and I19*Δ*mtri* for the MTAP, MTRI, and RuBisCO-MTRI knockout strains, respectively.

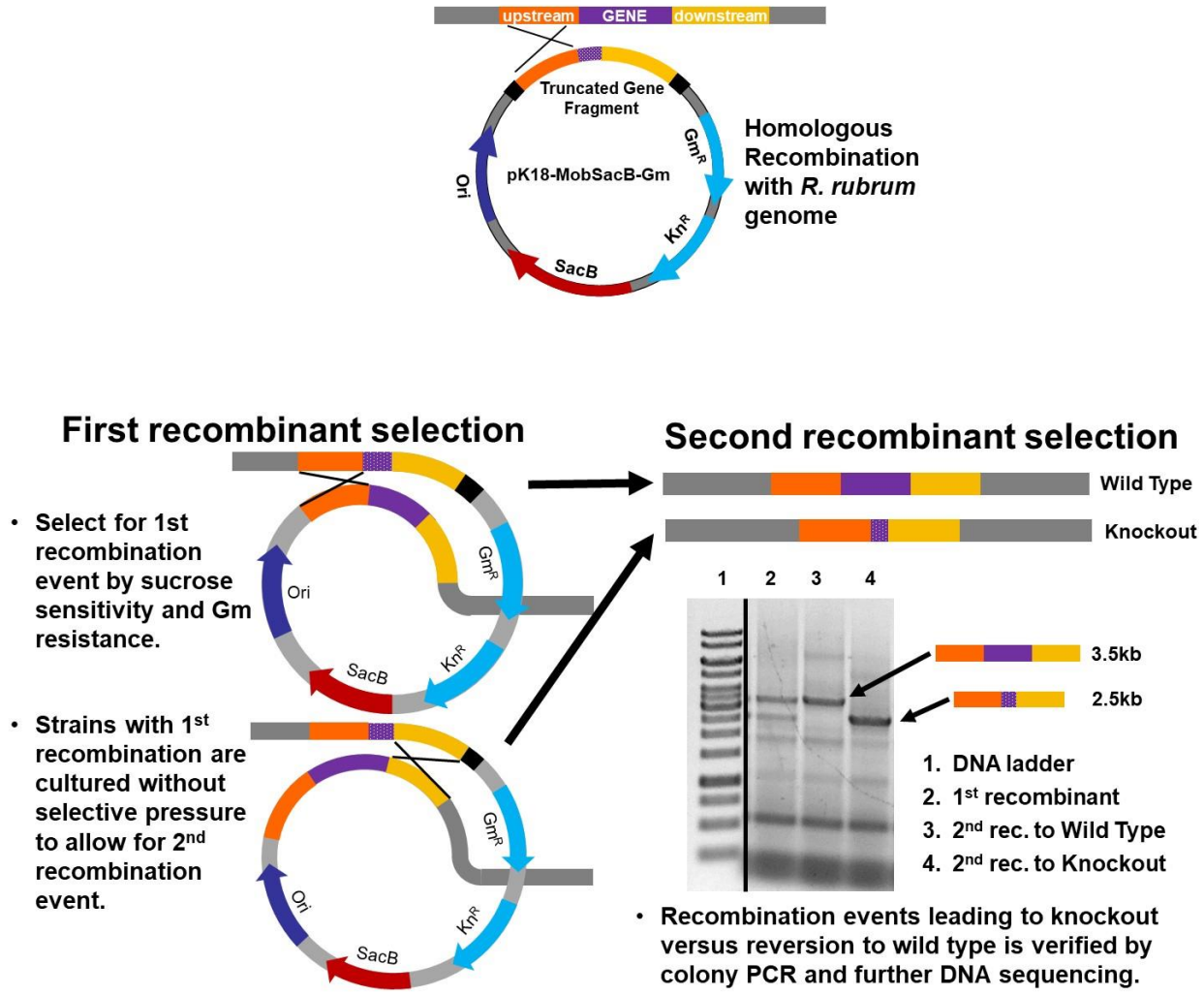

Figure 14: Gene deletion by homologous recombination. schematic of the construction and selection of the first and second recombinants in the construction of homologous recombination-mediated gene knockouts.

Construction of the MTAP and complementation plasmids

Insertion of *mtap* into pBBR1 to form pMTAP-RuMTAP

To insert the *R. mtap* gene into pBBR1, the *mtap* gene and putative upstream promoter element was amplified via PCR using two primers, 361F and 361R (Appendix I). These primers had restriction sites for AseI and XbaI respectively. The resulting PCR fragment was digest with AseI and XbaI and then ligated using T4 DNA ligase into pBBR1-MCS3 plasmid that had been digested with AseI and XbaI and Antarctic phosphatase treated. The resulting plasmid, pMTAP-RuMTAP, was able to express the phosphorylase gene from the native phosphorylase promoter when complemented in *R. rubrum* Δ*mtap* strain.

Construction of pMTAP for cloning of other genes

The ability of the putative *mtap* gene promoter element to drive expression of the *mtap* gene from plasmid pMTAP-RuMTAP suggested that a general complementation plasmid could be constructed. In order to construct the general complementation plasmid pMTAP for expression of exogenous genes in *R. rubrum*, the putative promoter for the *mtap* gene was amplified using two primers with restriction sites for AseI, labeled MtapF and MtapR for the forward and reverse primers respectively (Appendix I). The reverse primer also contained a restriction site for NdeI overlapping with the start codon of the phosphorylase gene. This fragment was digested using AseI (NEB), and ligated into pBBR1 base vector that had been digested using AseI and Antarctic phosphatase treated. The ligated vector was designated pMTAP due to the presence of the MTAP promoter. Importantly, since the NdeI site overlaps with the native start codon of the phosphorylase gene, any gene of interest can be ligated into this plasmid in the exact position as the native gene, given that it is amplified with a forward primer

that also utilizes an NdeI restriction site overlapping with the start codon of the gene. See Appendix I for primers and restriction enzymes used to amplify and clone *mtri* and various aldolase genes into pMTAP.

Construction of Δ*mtap+mtap* and Δ*mtri+mtri* complemented strains

The complementation vectors were then mated into their respective knockout strains using conjugative mating methods described above to form the Δ*mtap+mtap* and Δ*mtri+mtri* complemented strains.

Storage of strains

In order to allow the long-term storage *R. rubrum* strains, the cells were grown anaerobically in 10 mL of Omerod's Malate Minimal Media (MMM, Appendix II). When cells had reached mid-exponential phase, 0.8 mL of culture were taken and combined with 0.4 mL of Glycerol Storage Solution (GSS, Appendix II) into a 1.5 mL centrifuge tube (Axygen). The stock was then flash frozen in liquid nitrogen and stored at -80C.

Growth conditions of strains

R. rubrum strains from -80C stocks were streaked onto 16% agar-PYE plates using a four-phase streak to allow isolation of individual colonies. These plates were incubated at 30C until there were visible individual colonies, at which point single colonies were inoculated into 3 mL of liquid PYE in an aerobic culture tube. Strains with antibiotic resistances had the appropriate antibiotic added to the concentration listed in Appendix III to reduce the chance of contamination.

For aerobic cultures, 0.2 mL of the starter inoculum was sub-cultured into 5 mL of MMM with 1 mM SO_4 in aerobic culture tubes. When cells reached mid-exponential phase, 1 mL of culture was placed into a sterile 1.5 mL centrifuge tube and was washed three times. Each wash step entailed centrifuging the sample at 16000 xG for 2 minutes, followed by decanting of the supernatant and resuspension of the cell pellet in 1 mL of sulfur-free MMM. After the third wash, the cells were used to inoculate 10 mL of MMM to an initial optical density of OD_{660}~0.05 in aerobic culture tubes. Varying sulfur sources were added to the media based on the experiment being conducted, and are detailed in each section. Growth upon the sulfur source was measured by detecting the optical density of the culture (OD_{660}) over time.

For anaerobic cultures, 0.2 mL of the starter inoculum was sub-cultured into 10 mL of MMM with 1 mM SO_4 added as the sulfur source in anaerobic culture tubes. When cells reached mid-exponential phase, the culture was washed three times. Each wash step entailed centrifuging the anaerobic culture tube at 5000 xG for 5 minutes, followed by decanting of the supernatant and resuspension of the cell pellet in 10 mL of sulfur-free MMM. Note that all wash steps were conducted in an anaerobic chamber to minimize the presence of oxygen in the samples. After the third wash, the cells were used to inoculate 10 mL of MMM to an initial optical density of OD_{660}~0.05 in 28 mL anaerobic culture tubes. Varying sulfur sources were added to the media based on the experiment being conducted, and are detailed in each section. Growth utilizing each sulfur source was measured by detecting the optical density of the culture (OD_{660}) over time.

Section II: Chapter II methods

Deletion of *ald2*

The gene knockout of Rru_A0359, the putative aldolase gene (*ald2*) was done using homologous recombination, using methods described in Chapter V Section I and primers 359KF, 359SR, 359XF, and 359KR (Appendix I). This gene was knocked-out in the WT background to construct the Δ*ald2* strain and in the Δ*rlp* background to construct the Δ*rlp*Δ*ald2* strain. Furthermore, a complementation vector, pMTAP-ald2 was constructed using the pMTAP vector, using methods described in Chapter V Section I and primers found in Appendix I. This vector was transformed into *E. coli* SM10 and transferred into the Δ*ald2* and Δ*rlp*Δ*ald2* strains via conjugative mating to generate the complemented Δ*ald2+ald2* and Δ*rlp*Δ*ald2+ald2* strains. All primers that were used during this process are listed in Appendix I.

[^{14}C]MTA feedings of the *R. rubrum* strains

To see differences in metabolic profiles of *R. rubrum* gene deletion strains, cellular feedings with radiolabeled [^{14}C]MTA were conducted. [^{14}C]SAM was obtained from Perkin-Elmer and converted into [^{14}C]MTA using previously reported methodologies[5,12] by Justin North, a postdoctoral fellow certified to work with radioactive compounds. Cells of the desired *R. rubrum* strains were grown in MMM with sulfate as the sulfur source until they reached mid-exponential phase (OD ~0.5), washed three times with sulfur-free MMM, and then resuspended to a final OD ~0.3 with 5 μM [^{14}C]MTA and 100 μM unlabeled MTA present in the media. Timepoints of samples were taken at 0, 1, 2, 4, 6, 9, 13, 16 hours after feeding.

HPLC quantification of metabolites

Quantification of metabolites was done using a Shimadzu Prominence HPLC system. This system contained a separate inline β-RAM radiochromatography scintillation detector (IN/US Systems) which could be used to detect radiolabeled metabolites. This device furthermore had a dual-wavelength UV/Vis detection system that was used to measure absorbance of the samples at 215 nm and 260 nm. Elution of metabolites was achieved by use of a linear gradient from 0 to 50% acetonitrile (J.T. Baker) with 20 mM ammonium acetate buffer over 30 minutes at a temperature of 30C.

Purification of proteins

Proteins for the *in vitro* reconstitution of the putative *ald2* methionine salvage pathway were purified using the His-tag system. The selected proteins for purification were the gene products of the *R. rubrum mtap* gene (Rru_A0361), *Bacillus subtilis* methylthioribose kinase (*mtnK*) (which uses ATP to add a phosphate group to methylthioribose to form MTR-1P), *R. rubrum mtri* gene (Rru_A0360), and *R. rubrum ald2* gene (Rru_A0359).

The expression plasmids for synthesis of proteins MTAP, MtnK and MTRI had been previously described[5,12]. The expression plasmid for synthesis of *R. rubrum* Ald2 was constructed using pet28a, which contains the T4 bacteriophage RNA polymerase promoter, lac promoter, and the *lacI* gene for regulation of protein expression[34]. It also contains a 6His-tag site to add six histidine residues to the N-terminus of the gene of interest[34].

The *ald2* gene was cloned into the pET28a vector using methods similar to those described in Chapter V Section I to form the expression plasmid pET28-RuAld2. This was done by using the Ald2F and Ald2R primers (Appendix I) to amplify the gene fragment, which was

enzymatically digested with NdeI and SacI and ligated into pET28a which had also been digested with NdeI and SacI and Antarctic Phosphatase treated.

Expression vectors for the four genes were then transformed into chemically competent BL21 DE3 *E. coli* cells. These strains were grown until mid-exponential phase (OD ~0.8) at 37C in a shaking incubator. Protein synthesis was induced using isopropyl-β-D-1-thiogalactopyranoside, and cells were grown for an additional 12 hours at room temperature.

The cells were then centrifuged at 5000 xG for 10 minutes, and cells were lysed using a French Press pressure cell. The lysates were passed over a Nickel-NTA agarose resin (QIAGEN), which binds the His-tag of the recombinant produced proteins. The purified proteins were then eluted by displacement with imidazole, which has a chemical structure to the side chain of histidine. Purified enzyme was exchanged into storage buffer consisting of 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 50 mM KCl, and 10 % glycerol. This mixture was then flash-frozen using liquid nitrogen and stored at -80C.

In vitro assays with the ald2 gene product

In order to verify the function of the *ald2* protein product, MTRu-1P was enzymatically synthesized using previously reported protocols[5,12] utilizing purified MTR and enzymes MtnK, and MTRI.

MTRu-1P was then supplied to the *ald2* gene product. This was done in a 125 μL aliquot with 2 mM MTRu-1P, 5 mM $MgCl_2$, 25 mM Mops-KOH pH 7.5, and 20 μM of purified R. rubrum *ald2* gene product. This reaction was incubated at 30C for 2 hours, and then 1 μL of the product was analyzed via Gas Chromatography (GC) using a Shimadzu GC-14. The sample was run over an Agilent DB-wax column using helium as the carrier gas. Volatile compounds were

eluted from the column using a thermal gradient starting at 40 °C for 2 min followed by Δ10 °C/min to 180 °C. The compounds were detected using a flame-ionizing detector.

Growth experiments

Grown experiments for *R. rubrum* WT and Δ*ald2* were conducted using the same protocol as described in Chapter V, Section I, using either 1 mM ammonium sulfate or 1 mM MTOH as the sole sulfur source.

Section III: Chapter III methods

Construction of an ald2 complementation system

The complementation plasmid for *ald2* genes was constructed using the pMTAP plasmid. Transgenic aldolases were inserted into the pMTAP vector using methods described in Chapter V Section I. The following vectors were constructed with their respective primers: pMTAP-RpAld2 using primers 4655F and 4655R, pMTAP-ElAld2 using primers 02190F and 02190R, pMTAP-MmAld2 using primers 15895F and 15895R, pMTAP-EcFucA using primers 1831F and 1831R, and pMTAP-EcAld2 using primers 2396F and 2396R. pMTAP-ald2 was constructed using primers and restriction enzymes as listed in Appendix I.

These vectors were mated into the *R. rubrum* Δ*rlp*Δ*ald2* background using methods described in Chapter V Section I. In addition to the preexisting Δ*ald2+ald2* strain described in Chapter V Section II, this same strategy was used to form the Δ*ald2+Rpald*, Δ*ald2+Mmald*, Δ*ald2+Elald*, Δ*ald2+EcfucA*, and Δ*ald2+Ecald* strains, which are complemented with respective putative *ald2* genes from *R. palustris, M. morganii, E. limosum, E. coli* ATCC 25922.

Growth conditions of *R. rubrum* complemented strains

The complemented strains were grown in triplicate anaerobically as described in Chapter V Section I above. All cultures had 10 mL of media, leaving 18 mL of headspace from which ethylene was collected. 0.25 mM MTA was used as the sulfur source for all of the complemented strains.

Quantification of ethylene production

Ethylene production was quantified by GC using a Restek Rt-Alumina BOND/Na_2SO_4 column connected to a Shimadzu GC-14C instrument with flame ionization detection. Helium carrier gas was maintained isothermally at 30C. Samples were analyzed approximately 72 hours after the cells had reached stationary phase. The amount of ethylene detected was quantified and is reported as the total amount of ethylene produced in micromoles per liter culture per Optical Density (μmol/L/OD_{660}).

Growth conditions of *M. morganii* INSali207, *E. limosum* ATCC 8486, *E. coli* ATCC 25922, *R. palustris* CGA010, and *R. rubrum* ATCC11170

M. morganii INSali207 was grown using Morganella media (Appendix II). Sulfur sources were added in the following amounts: 1mM methionine, 1 mM cysteine hydrochloride, 0.25 mM MTA. *M. morganii* was grown in 10 mL anaerobic cultures at 37C. Growth was measured as a +, +/-, or - phenotype. Growth media was formulated in the Tabita lab, but as *M. morganii* is a BSL-2 pathogen, all culturing was generously done by the Wozniak lab (Ohio State).

E. limosum ATCC 8486 (generous gift from J.A. Krzcycki, Ohio State) was grown upon Eubacterium media (Appendix II). Sulfur sources were added in the following amounts: 1 mM cysteine hydrochloride, 0.25 mM MTA, 1 mM MTOH. *E. limosum* was grown in 10 mL anaerobic cultures at 37C. Growth was measured as a +, +/-, or - phenotype.

E. coli ATCC 25922 (American Type Culture Collection) was grown upon M9 media (Appendix II). Sulfur sources were added in the following amounts: 1 mM cysteine hydrochloride, 1 mM ammonium sulfate, 0.25 mM MTA, 1 mM MTOH. *E. coli* was grown in 10 mL anaerobic cultures at 37C. Growth was measured as a +, +/-, or - phenotype.

R. palustris CGA010 was grown upon Ormerod's Malate Minimal Media with added para-aminobenzoic acid (MMM+pABA, Appendix II). Sulfur sources were added in the following amounts: 1 mM cysteine hydrochloride, 1 mM ammonium sulfate, 0.25 mM MTA, 1 mM MTOH, 1 mM methionine. *R. palustris* was cultured under both aerobic and anaerobic conditions in 10 mL cultures at 30C. Growth was measured as a +, +/-, or - phenotype.

R. rubrum ATCC 11170 was grown upon MMM as described before. Sulfur sources were added in the following amounts: 1 mM cysteine hydrochloride, 1 mM ammonium sulfate, 0.25 mM MTA, 1 mM MTOH, 1 mM methionine. *R. rubrum* was cultured under both aerobic and anaerobic conditions in 10 mL cultures at 30C. Growth was measured as a +, +/-, or - phenotype.

Section IV: Chapter IV methods

Quantification of excreted 5dAdo in *R. rubrum* and *R. palustris* knockout strains

In order to quantify 5'-deoxyadenosine excretion in *R. rubrum* and *R. palustris*, knockout strains of the organisms were grown in triplicate in MMM or MMM+pABA (Appendix II) respectively with 1mM SO_4 as the sulfur source. Strains were grown under both aerobic and anaerobic conditions, as described in Chapter V Section I.

The strains used for *R. rubrum* were the Δ*mtap*, Δ*mtap+mtap*, Δ*mtri*, Δ*mtri+mtri*, Δ*ald2*, Δ*ald2+ald2*, Δ*rlp*Δ*ald2*, Δ*rlp*Δ*ald2+ald2*, Δ*rlp*, and WT strains. Samples of spent media were taken at 0, 48, 72, 96, 120, and 144 hours. Strains not previously discussed in this thesis had been constructed previously by other members of the Tabita lab (see 5,12, and unpublished work by J.A.N and A.R.M).

The strains used for *R. palustris* were the Δ*mtap*+empty vector, Δ*mtap+mtap*, Δ*mtri*+empty vector, Δ*mtri+mtri*, Δ*rlp1*Δ*ald2*, Δ*rlp1*Δ*ald2+ald2*, Δ*rlp1*, Δ*rlp2*, and WT strains, and samples of spent media were taken at 0, 24, 48, 72, 96, 120 hours. All *R. palustris* strains had been previously constructed by other members of the Tabita lab (see 5,12, and unpublished work by J.A.N and A.R.M).

Samples were centrifuged for 7 minutes at 16,000 xG to pellet the cells, and 200 μL of the supernatant was run over the HPLC using the Agilent Zorbax C18 column and buffer system described in Chapter V, Section II.

5'-deoxyadenosine comes off as a distinctive peak at ~27 minutes under this buffer system, and can be detected using UV/Vis absorbance at 260 nm. The absorbance of the samples

was measured, and was correlated to concentration of 5'-deoxyadenosine based on a standard curve.

Reconstitution of the putative 5dAdo salvage pathway in vitro

In order to detect metabolites, [^{3}H]5'-deoxyadenosine was obtained from Moravek Biochemicals. While I could contribute to the maintenance of strains, preparation of enzymatic systems, and data analysis, all handling of radioactive materials was done by a postdoctoral fellow, Justin North, who was certified for radioactivity work.

The proteins used in this experiment were MTAP, MTRI, and RLP from *R. rubrum*, RLP1 and RLP2 from *R. palustris*, and DXP reductoisomerase (DRI) from *E. coli* ATCC 25922. The purification of MTAP and MTRI have been previously described. RLP, RLP1, and RLP2 proteins were obtained from previous preparations by other members of the Tabita lab (see 5,12, and unpublished results by J.A.N and A.R.M). Synthesis and purification of DXP reductoisomerase was carried out in a similar manner as described in Chapter V Section II. pET28-DRI was constructed by amplifying the *E. coli dri* gene using primers DRIF and DRIR (Appendix I), digesting with NdeI and BamHI, and ligating using T4 ligase into pET28a plasmid digested with the same enzymes and Antarctic phosphatase treated.

Samples were reacted in 100 μM 5dAdo, 0.1uCi [^{3}H]5dAdo, 50mM HEPES 7.5, 25mM K-phos 7.5, 5 mM $MgCl_2$, 10mM DTT with 20 μM MTAP and 20 μM MTRI. To this, either 20 μM of *R. rubrum* RLP, 10 μM *R. palustris* RLP1, or 10 μM *R. palustris* RLP2 was added. When adding DRI to these reactions, 20 μM DRI and 0.4 mM NADPH was also included.

Reactions were incubated for 2 hours at 30C, and then quenched with 170 μL of acetonitrile. Addition of acetonitrile led to the formation of an insoluble fraction, which was

eliminated by centrifuging the sample at 10000 xG for 5 min and collecting the supernatant. 200 μL of the supernatant was then run over HPLC using a SeQuant zic-pHILIC column designed to bind and separate hydrophilic compounds with the following protocol: 80% acetonitrile for 5 minutes, linear decrease to 40% acetonitrile over 33.33 minutes, linear increase to 80% acetonitrile over 10 minutes, all in 10mM ammonium bicarbonate pH 9.2. Coordinately the flow rate was maintained at 0.35mL/min for 15 minutes, followed by linear decrease to 0.25mL/min over 2 minutes, hold for 38 minutes, then linear increase over 2 minutes to 0.35mL/min, hold until a total of 70 minutes.

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(J.A.N. unpublished results) refers to unpublished results from Justin A. North, a postdoctoral fellow in the Tabita lab at the time of this thesis.

(A.R.M. unpublished results) refers to unpublished results from Anthony R. Miller, a graduate student in the Tabita lab during the time of this thesis.

Appendix

I : Primers used

Primer Description	Primer Name	Fragment #	Sequence (5' - 3')	Fragment R.E.*	Plasmid R.E.†
Construction of pK18-RuMtnP from pK18mobsacB	361KF	1	CGAGGAGCCCCTCGAGACCATCACCCAGCTCGC	XhoI	SalI
	361XR	1	CCCGATCCGCCGATTCTAGAAAGCACCGGCTGC	XbaI	--
	361SF	2	GGATGGCGCGCAAAACTAGTGCCGTCGCCGGTCGG	SpeI	--
	361KR	2	GCAAACGCCGCATGCGGTGATCAAGCCGGTGATCA	SphI	SphI
Construction of pK18mobsacBgm from pK18mobsacB	GenF	1	CGGTTCGTAAACTGTAATGCTAGCAGCGTATGCGCTCAC	NheI	NheI
	GenR	1	CGCTGCAAGAAGGCTAGCTGGTGGCGCTTGC	NheI	NheI
Construction of pK18-RuAld2 from pK18mobsacBgm	359KF	1	CCCGCTTGGGCTCGAGTTTTACAAGGATGATCG	XhoI	SalI
	359SR	1	CCGGGCGGCAACTAGTAAACCATGACGCAGGG	SpeI	--
	359XF	2	CAAAACCTATCTAGATAACGCCCAGCTCCCCTC	XbaI	--
	359KR	2	GCACAAGGCGGCATGCCAGAGGACAAGGCGGC	SphI	SphI
Construction of pMTAP-RuMtnP from pBBR1-MCS3	361F	1	GCGACGTCAAGCATTAATAAACCGCTTTAACCGC	AseI	AseI
	361R	1	GGGTAGGAGTGCTCTAGACGTTCATCGGCCGATCA	XbaI	XbaI
Construction of pMTAP-MCS3 from pBBR1-MCS3	MtapF	1	GCGACGTCAAGCATTAATAAACCGCTTTAACCGC	AseI	AseI
	MtapR	1	ACTCACATTAATTGCGTTCATATGTTCCCCTCTTCCTGGCTGGCT	AseI	AseI
Construction of pMTAP-RuMtnA from pMTAP-MCS3	360F	1	GATGATCGGCATATGAACGTCAAAGGCACTCC	NdeI	NdeI
	360R	1	GATCCGCGATTCTAGAATCAGAGCGCCCCGTCG	XbaI	XbaI
Construction of pMTAP-RuAld2 from pMTAP-MCS3	359F	1	GGCGCTCATATGCCCGGATCGCG	NdeI	NdeI
	359R	1	CGACGGACGTCTAGATGGGGGAGTCTCCTTGG	XbaI	XbaI
Construction of pMTAP-RpAld2 from pMTAP-MCS3	4655F	1	GGAAACAAACATATGACGCTTCCCGGCGCTGC	NdeI	NdeI
	4655R	1	GATGGGCTCTAGAGCAAGCCTTGCGCAGTGTTGG	XbaI	XbaI
Construction of pMTAP-MmAld2 from pMTAP-MCS3	15895F	1	CTGAGTATGAGGTGTGCATATGACCCGTACTGAACTGG	NdeI	NdeI
	15895R	1	TACGTAAGGTGACAAATCTAGACGCATTGCGCCG	XbaI	XbaI
Construction of pMTAP-ElAld2 from pMTAP-MCS3	02190F	1	GACGATTTTCTCATATGAGTAAACCCCATCAAAACCCGGAGG	NdeI	NdeI
	02190R	1	AACTTAGCTTCTAGAGTTCTTAAGGCTTGGGGCACGC	XbaI	XbaI

Construction of pMTAP-EcFucA from pMTAP-MCS3	1831F	1	TCAGAGAGAGGTAATTAATGGAACGAAATAAACTTGCTCGTC	AseI	NdeI
	1831R	1	GCCCCAACAGCATCTAGACCAAACCATGCCGTTTC	XbaI	XbaI
Construction of pMTAP-EcAld2 from pMTAP-MCS3	2396F	1	GCTATTAAGGATCACATATGGAACGGATTAAGTTAGC	NdeI	NdeI
	2396R	1	GCGAATGGCTGTCTAGAGTTATTAATTAAAATAAAGC	XbaI	XbaI
Construction of pET28-RuAld2 from pET28a	Ald2F	1	GGCGCTCATATGCCCGGATCGCG	NdeI	NdeI
	Ald2R	1	GGAAAGCCCGAGCTCTTGGCAAGCCTCG	SalI	SalI
Construction of pET28-EcDRI from pET28a	DRIF	1	CTCTGGATGTCATATGAAGCAACTCACCATTCTGGGCTCG	NdeI	NdeI
	DRIR	1	GACTATATCACTGGATCCCTACGCTAACAAATAGCGCGAC	BamHI	BamHI

II : Growth media used

Growth media has been previously described and/or cited in sources (5), (12).

Ormerod's Malate Minimal Media (MMM)

1000x Sulfate Free Trace Elements
10 mM $NiCl_2 \cdot H_2O$
12.5 mM $MnCl_2 \cdot 4H_2O$
45 mM H_3BO_3
1.6 mM $CuCl_2$
3 mM $NaMoO_4$
0.9 mM $CoCl_2$

10x Sulfate Free Basal Salts:
400 mM KCl
10 mM $MgCl_2$
5 mM $CaCl_2$
0.25 mM $FeCl_3$
0.35 mM $Zn(CH_3O_2)_2 \cdot 2 H_2O$
0.6 mM EDTA $\cdot 2 Na \cdot 2H_2O$
1% v/v 1000x Sulfate Free Trace Elements

Vitamin solution
1 mg/mL thiamin HCl
1 mg/mL nicotinic acid
All in 50% ethanol

Biotin solution
10 % w/v Biotin in 50% ethanol

MMM
10% v/v Sulfate Free Basal salts
10 mM NH_4Cl
0.015% v/v Biotin solution
0.1% v/v Vitamin Solution
30 mM DL-malate

MMM+pABA

Identical to MMM, but with added 2 μg/mL para-aminobenzoic acid

PYE

0.3% w/v Peptone
0.3% w/v Yeast Extract
10% v/v Basal Salts (see MMM for details)
0.1% v/v Vitamin solution (see MMM for details)
0.015% Biotin solution (see MMM for details)
pH to 6.8

GSS

65% w/v glycerol
0.1M $MgSO_4$
25 mM Tris pH 7.6

LB

1% w/v Tryptone

0.5% w/v Yeast Extract
0.5% w/v NaCl

CSOC

0.6% w/v Peptone
0.5% w/v Yeast Extract
0.058% w/v NaCl
0.0186% w/v KCl
10% v/v Basal Salts (see MMM for details)
0.1% v/v Vitamin solution (see MMM for details)
0.015% Biotin solution (see MMM for details)

SOC

0.6% w/v Peptone
0.5% w/v Yeast Extract
0.058% w/v NaCl
0.0186% w/v KCl

M9

M9 media (10ml)
2ml 5x M9 salts
200ul 50X Mg/Ca
200ul 50X Ormerod's trace elements
250ul 1M glucose
S-source
8ml water

5x M9 salts
31g Na_2HPO_4 (425mM)
15g KH_2PO_4 (110mM)
2.5g NaCl (43mM)
5g NH_4Cl (93mM)
5mg/L thiamin and nicotinic acid (5ml of 1mg/ml solution)
deionized H_2O, to 1 liter
Autoclave and store at RT. (total monovalent cationic strength 1x = ~130mM)

50x Mg/Ca
2.033g $MgCl_2*6H_2O$ (0.1M)

500uL 1M CaCl2*2H2O (5mM)
Deionized H2O to 100ml.
Autoclave and store at RT.

1M Glucose
Make 100ml of 1M glucose. Filter sterilize into autoclaved container

50X Ormerod's Trace Elements
5ml 1000x ormerod's trace elements
0.034g FeCL3 * 6 H2O (270.30 g/mol, 1.25mM)
0.6ml 0.5M EDTA (3mM)
0.038g Zn(CH3O2)2*2H2O (219.51 g/mol, 1.75mM)
Deionized water to 100ml. Filter sterilize into autoclaved container.

Morganella Media

Composition (in g/l) of the SP medium was as follows:

Salts
NaCl: 0.5
KCl: 0.4
MgSO4-7H20: 0.2 (can replace with MgCl2-6H2O)
CaCl2-2H2O: 0.265
NH4Cl: 0.535

Amino Acids
L-arginine: 0.126
L-glutamine: 0.292
L-histidine: 0.042
L-isoleucine: 0.052
L-leucine: 0.052
L-lysine: 0.0725
L-phenylalanine: 0.0325
L-threonine: 0.048
L-tryptophan; 0.01
L-tyrosine: 0.036
L-valine: 0.046

Vitamins
biotin: 0.001
folic acid: 0.001
nicotinic acid: 0.001
pantothenic acid: 0.001
pyridoxal: 0.001

riboflavin: 0.0001
thiamin: 0.001.

pH to 7.2 with K-phosphate.

Elim media

Media recipe amalgamated and modified by Justin North.

Justin's Modified Bryant and Pfenning (Based on 1 and 2)

1) SHARAK GENTHNER, C. L. DAVIS, AND M. P. BRYANT. Features of Rumen and Sewage Sludge Strains of Eubacterium limosum, a Methanol- and H2-C02-Utilizing Species. APPLED AND ENVIRONMENTAL MICROBIOLOGY, July 1981, p. 12-19
Modified Pfenning for Growth of E.lim ATCC 8486

2) Michael J. McInerney, Marvin P. Bryant, and Norbert Pfennig. Anaerobic Bacterium that Degrades Fatty Acids in Syntrophic Association with Methanogens. Arch. Microbiol. 122, 129-135 (1979) **Original Bryant and Pfenning medium**

3) John A. Breznak, Jodi M. Switzer, and H.-J. Seitz. Sporomusa termida sp. nov., an H2/CO2-utilizing acetogen isolated from termites. Arch Microbiol (1988) t 50 : 282- 288. **Original AC-21 Medium Recipe**

4) M. Lelait and J. P. Grivet. Carbon Metabolism in Eubacterium limosum : a 13C NMR Study. Anaerobe (1996) 2, 181–189. **Modified AC-21 medium for E. limosum**

Basal Medium: (From 1)

50ml 20x Pfennings Mineral Solution
1ml Pfenning's 1000x Metal Solution (note Krzycki lab tripled for E. lim growth)
0.1ml 10000x Selenite/Tungstate solution (from 3)
5ml Vitamin solution

1ml 0.1% Resazurin (0.0001% final)

Bring volume up to 1L

Make anaerobic on the manifold using anaerobic N2 gas.

Autoclave 121C for 20 minutes, then when cooled, pass into anaerobic chamber under 5%/95% H2/N2.
Dispense into sterile Crimp Cap Tubes then add the following which is filter-sterile and anaerobic:

- For Chemoheterotrophic growth under N2 atmosphere add: (From 1)
  -0.05ml/ml culture 1M K-phos pH 7.2 (50mM)
  -0.02ml/ml culture of 1M glucose (20mM final) and 0.01ml/ml culture 2M sodium acetate (20mM final)
  -OR 0.04ml/ml culture of 0.5M Mannitol (20mM Final)

Then Add:

-0.0075ml/ml culture of 0.2M Cysteine-HCL (1.5mM Final)
-0.0075ml/ml culture of 0.16M Na2S (1.2mM Final)
OR
-0.01ml/ml culture of 1M sulfur source (cys, met, SO4, 1mM final)
-Titanium Citrate solution

Wait serveral hours then add culture

Crimp, pass out of anaerobic chamber, and put under appropriate atmosphere listed above.

<u>20x Pfennings Mineral Solution: (From 2)</u>

NH_4Cl	8g
MgCl2 x 6 H2O	6.6g
$CaCl_2$ x 2 H_2O	1.0g
NaCl	8g
KH2PO4	10g
ddH2O	1L

Add to water and store in the dark at RT

<u>1000x Pfennig Metal Solution: (From 2)</u>

Zn(CH3O2)2 x 2 H2O	0.076g
MnCl2 x 4 H2O	0.03 g
H3BO3	0.3 g
CoCl2 x 6 H2O	0.20 g
CuCl2 x 2 H2O	0.01 g
NiCl2 x 6H2O	0.02 g
Na2MoO4 x 2 H2O	0.03 g
FeCl3 x 6 H2O	2.0 g
EDTA x 2 Na+2 H2O	5.0 g
ddH_2O	1L

Add boric acid and EDTA first, pH 3.0-4.0 with HCL to dissolve then add everything else. Wrap in foil and store in the dark at RT.

10000x Selenite/tungstate solution: (from 3/4)

NaOH	0.2ml (10N)
Na2SeO3 x 5 H2O	2.6 mg
Na2WO4 x 2 H2O	3.3 mg
ddH_2O	Volume to 100ml

Add to water. Filter sterilize and place under N2 gas. Wrap in foil store at RT in the dark.

1M Phosphate Buffer 7.2

44g/L	KH2PO4
119g/L	K2HPO4

Bring volume up to 1L and autoclave.
Keep in anaerobic chamber

Cysteine-Na2S reducing solution

1.92g Na2S x 9 H2O (240.18g/mol) 0.16M
Bring volume up to 50ml. Filter sterilize and place under N2 gas.
Keep in anaerobic chamber.

1.57g Cys-HCL (157.6g/mol) 0.2M
Bring volume up to 50ml. Filter sterilize and place under N2 gas.
Keep in anaerobic chamber.

-Add separately to media when ready.

200x Vitamin Solution: (From 1)

Biotin	2mg	R. rub, R. pal, E. lim required
Folic Acid	2mg	
Pyridoxal HCL	1mg	
Lipoic Acid	6mg	E. lim required
Riobflavin	5mg	
Thiamin HCL	5mg	R. rub, R. pal given but not required
Ca-D-pantothenate	5mg	E. lim required
cyanocobalamin	5mg	
p-amino-benzoic acid	5mg	R. pal required
nicotinic acid	5mg	R. rub, R. pal given but not required
ddH2O	100ml	

Add 20ul of 10N NaOH to bring pH to ~8.0 to get all solids to dissolve.
Filter sterilize and place under N2 gas. Store at -20C and take in anaerobic chamber as needed.

Titanium Chloride Solution (Joe Krzycki)
0.15 ml TiCl3 in HCL (Sigma)
0.13 ml 2 M Na-citrate buffer (pH 8)
0.425 ml Tris base near saturated solution (around 4 M)
295 ul dH20.

III : Antibiotic concentrations

***R. rubrum* and *R. palustris*[5,12]**

1000x stocks:
Streptomycin: 50 mg/mL
Gentamycin: 30 mg/mL
Kanamycin: 50 mg/mL
Tetracycline: 2-3 mg/mL

E. coli

1000x stocks:
Streptomycin: 100 mg/mL
Gentamycin: 15 mg/mL
Kanamycin: 50 mg/mL
Tetracycline: 4 mg/mL
Ampicillin: 50 mg/mL
Chloramphenicol: 35 mg/mL